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APPROVED LABORATORY
TECHNIC

CLINICAL PATHOLOGICAL, BACTERIOLOGICAL, SEROLOGICAL, BIOCHEMICAL, HISTOLOGICAL

Prepared under the Auspices of

THE AMERICAN SOCIETY OF CLINICAL PATHOLOGISTS

BY

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With eleven colored plates and three hundred illustrations in the text

D. APPLETON AND COMPANY
NEW YORK   LONDON

Dedicated

to

GEORGE H. MEEKER, Ph.D., D.D.S., Sc.D., LL.D.

PROFESSOR OF BIOCHEMISTRY AND DEAN, GRADUATE SCHOOL OF MEDICINE,
UNIVERSITY OF PENNSYLVANIA

and in memory of

WARD BURDICK, M.D.

FOUNDER OF THE AMERICAN SOCIETY OF CLINICAL PATHOLOGISTS

PREFACE

It is hoped that this manual will aid in the fulfillment of several of the objects of the American Society of Clinical Pathologists, namely, to establish standards for the performance of various laboratory examinations, to promote the practice of scientific medicine by a wider application of clinical laboratory methods to the diagnosis of disease and to encourage a closer coöperation between the practitioner and the clinical pathologist.

In the preparation of this edition, however, the authors have assumed responsibility for the selection of the methods given and therefore these can not be stated to have the approval of the Society as a whole. But the description of the technic of each method has been definitely approved by at least five members of the Society so that the title finally chosen for this edition of the book is "Approved Laboratory Technic, Prepared under the Auspices of The American Society of Clinical Pathologists."

In exercising a special effort to strike a proper balance in the amount of detail given to fulfill the needs of the laboratory technician without being burdensome to the expert, even the simplest details are frequently given with the hope that these will insure greater accuracy and uniformity in results and inspire greater confidence by physicians in laboratory examinations conducted by A. S. C. P. Approved Technic. And since there is a growing and gratifying increase in the use of laboratory methods by practicing veterinarians in the diagnosis of diseases of the lower animals, an effort has been made to render the manual of equal service to them.

It is realized that no amount of detail or simplicity of presentation can make up for the deficiencies of inexperienced, careless and incompetent technicians, but it is believed that the descriptions and illustrations are adequate for insuring accurate work by experienced and careful workers and for the teaching of clinical pathology.

Throughout the book an effort has been made to emphasize the importance of using accurate and reliable apparatus and reagents, as not infrequently the results of very careful and painstaking examinations and analyses are rendered worthless by inaccuracies in these particulars. For example, if blood counting pipets and counting chambers are inaccurately calibrated or broken, the counts can not be even approximately correct despite great care exercised in making them; if the antigen and other biological reagents employed in the complement-fixation test for syphilis are lacking in sensitiveness, the reactions can not be reliable regardless of the care and skill exercised in setting up the tests. Under such conditions any method may be very precise and the worker very careful,

but the results quite inaccurate and misleading. It is fitting and proper, there-
fore, to lay particular emphasis upon these and other sources of error.

Special emphasis has been placed upon quantitative tests and reactions, since
these tend to greater accuracy and render more nearly possible approximately
similar results from different laboratories. And in qualitative tests an effort
has been made to suggest a uniform terminology and methods for reporting
reactions.

Since laboratories are frequently required to secure specimens of blood,
spinal fluid, gastric contents, bile, etc., for examination, methods for obtaining
these are included.

While the field of "clinical pathology" is difficult to define, yet in practice
it has come to include not only methods for the examination of blood, urine,
feces, sputum, etc., but likewise those bacteriological, serological and chemical
methods ordinarily requested in medical laboratories. Therefore, these sub-
jects are included. Histological methods may be omitted and for this reason
are not considered with the same amount of detail, although it is particularly
gratifying to be able to include a chapter on Methods for the Microscopical
Examination of Tissues by Dr. William C. MacCarty and Dr. W. L. A.
Wellbrook.

The authors are particularly indebted to Mr. Alexander Keller, Jr., for
assistance in reading and correcting the proofs of the section on Chemical
Methods and for permission to use a number of methods from the Manual of
the Biochemical Laboratories of the Graduate School of Medicine of the Uni-
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Exton and Dr. Anton Rose, is acknowledged; also, to Dr. Elizabeth Yagle for
assistance in preparing the section on Serological Methods and to Dr. Henry
L. Bockus for assistance in preparing the chapters on the examination of
stomach and duodenal contents and bile. And we especially beg to express
deep appreciation of the unselfish, highly efficient, painstaking and prompt
assistance rendered by the Committees of the Society whose names are grate-
fully given on the title page and without whose cooperation the book would
not have been possible, as well as deep appreciation of the unvarying courtesy
and efficiency of the publishers.

Philadelphia.

J. A. K.
F. B.

NOTE

THE *technic* of the methods contained herein has been approved by Committees of the American Society of Clinical Pathologists.

But there has been no attempt at present on the part of the Society to approve or recommend any of the *methods* for the diagnosis of disease. The authors alone assume full responsibility for the selection of these and in some instances have included two or more for the same examination, when it was impossible to decide upon one and when it was considered advisable to furnish alternate methods for controls.

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APPROVED LABORATORY TECHNIC

SECTION I

GENERAL LABORATORY METHODS

CHAPTER I

THE USE AND CARE OF THE MICROSCOPE AND METHODS OF MICROMETRY

EQUIPMENT

There is probably no laboratory instrument whose usefulness compares with that of the microscope, none depends so much upon proper manipulation and care, and none is more frequently abused and misused by both beginners and experienced workers.

1. A number of excellent instruments are available, made by Bausch and Lomb, Spencer, Leitz, and Zeiss, the last two being of foreign manufacture. It is poor economy to buy a cheap one.

2. The microscope should be of the side-arm type with a large stage. It should be provided with a substage condenser, at least three objectives on a triple nosepiece and two eyepieces (see Fig. 1).

3. Objectives are of two classes, achromatic and apochromatic. Those in general use are of the achromatic type, and they fulfill all requirements for ordinary work. Apochromatic objectives are more highly corrected for chromatic and spheric aberration, and represent the highest type of microscope lenses produced. They give crisp images with little or no trace of the color fringes which with achromatic objectives can readily be seen about the edges of black or colorless objects lying in a bright field, and are hence very desirable for photomicrography and research. They are also recommended for routine laboratory work and particularly for the examination of unstained objects, providing their cost is no obstacle. Within recent years, by use of fluorite in conjunction with special glasses, a third type of objective whose color corrections and price are midway between the achromats and apochromats, has been constructed. This is sometimes designated as a "semi-apochromat."

4. The simple eyepieces which have long been used with achromatic objectives are known as huygenian oculars. With apochromatic objectives it is necessary to use special "compensating eyepieces" which are corrected to overcome certain defects in this type of objective. The same compensating eyepiece may be used with oil-immersion and high dry objectives of the achro-

matic series.   A third type of ocular has recently been introduced under the trade names "hyperplane," "periplan," and so forth.   They have a compensation midway between the compensating and huygenian eyepieces and may

....................*E*

....................*D*

R....................   ....*T*
PH....................

MH... ....

....................*RN*

....................*O*

H....................

. ...*S*

I....................   ....*SS*

P....................   ....*M*

B....................
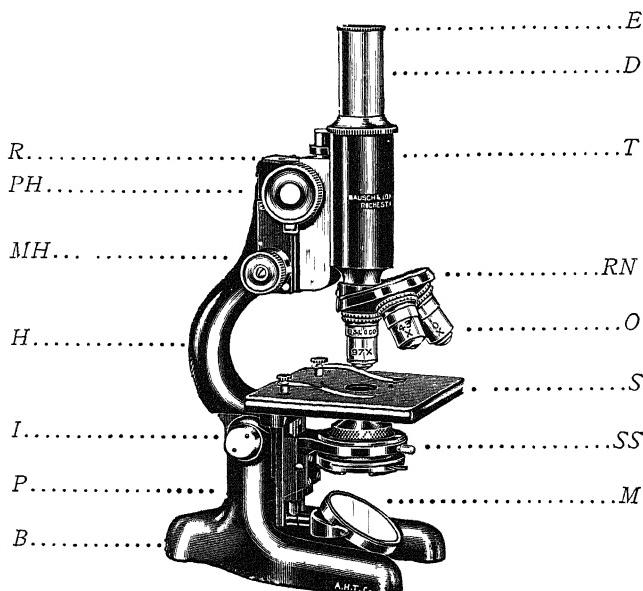
FIG. 1.—A SATISFACTORY TYPE OF MICROSCOPE

E is the eyepiece, of which two or three are usually furnished for varying degrees of magnification.

D is the draw tube, which is calibrated and should always be drawn to 160 or any other length recommended by the manufacturer.

T is the body tube

RN is the revolving or triple nosepiece carrying the objectives.

O is one of the usual three objectives.

R is the rack upon which the tube is raised on lowered.

PH is the pinion screw for coarse adjustment.

MH is the micrometer screw for fine adjustment.

H is the handle.

S is the stage.

SS is the substage carrying the Abbé condenser with diaphragm.

M is the mirror with plane and concave surfaces.

I is the inclination joint for using the microscope in an inclined position.

P is the pillar.

B is the base, which should be large and solid.

be used with either achromatic or apochromatic objectives.   Their chief advantage is that they overcome to a very marked degree the curvature of field of any objective with which they may be used.

5. A mechanical stage, preferably of the detachable type, is almost indispensable and is highly recommended (Fig. 2).

6. The binocular microscope (Fig. 3) of the new single objective type is recommended as it gives an impression of stereoscopic vision with flat fields

and sharper images, and enables the worker to keep both eyes open with less strain. The distance between the eyes is adjustable and the left eyepiece may be focused for adjusting any slight refractive differences between the eyes. They cost more than the ordinary microscope but are worth the difference, especially for tissue examinations, differential blood counting and bacteriological work. At the present time binocular tube attachments are procurable by which the ordinary monocular microscope may be transformed into a binocular. That shown in Figure 4 may be particularly recommended as it is built with inclined oculars insuring a correct and comfortable position with a horizontal stage for the specimen.

FIG. 2—MECHANICAL STAGE

7. The euscope (Fig. 5) has advantages not offered by binocular microscopes because it is not only the least fatiguing of all microscopic devices but enables very simple photography and is being widely used for demonstrations to small classes. Euscopy is done by projection, with all its attendant facilities and advantages; as a result, routine microscopy is brought, as regards comfort
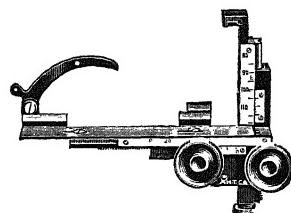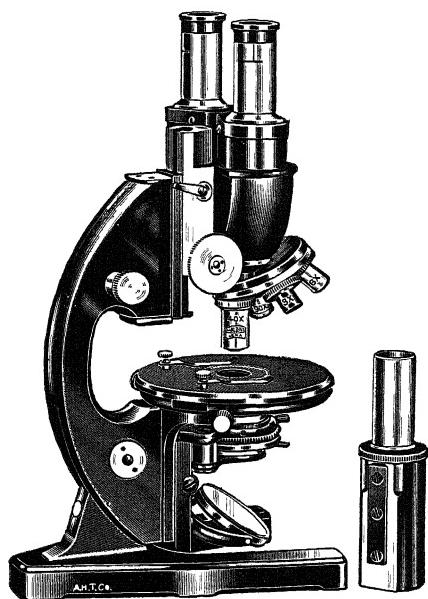
FIG. 3.—BINOCULAR MICROSCOPE
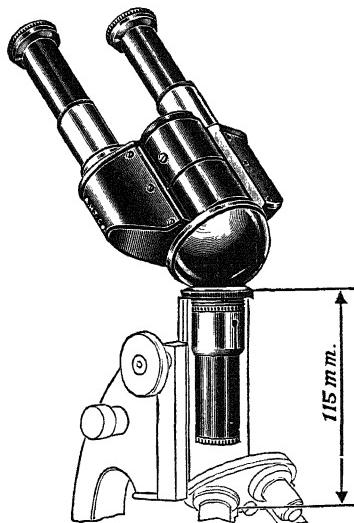
FIG. 4.—BINOCULAR TUBE ATTACHMENT.

and strain, to a level with ordinary clerical work and increases the efficiency and capacity of the microscopist.

8. A dark-field outfit is essential for examinations for *Spirochaeta pallida* and other spirochetes in a living state. It constitutes the so-called ultramicroscope, which is a misnomer because it does not magnify any higher than the

ordinary outfit but renders visible very minute particles by adopting the principle of blocking out the central rays of light and directing the peripheral rays against the object from the side.

A special condenser (Fig. 6) is required. Types are now available which may be placed in the stage of the microscope after removing the ordinary Abbé condenser.

A funnel stop is also required to reduce the
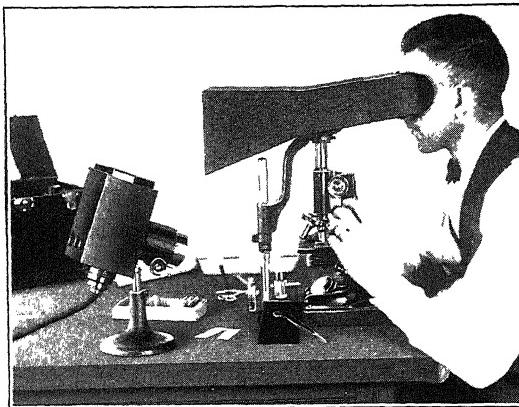
FIG 5.—SIMPLIFIED EUSCOPE, EXTENDED, WITH ACCESSORIES
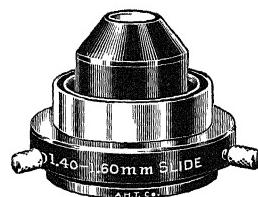
FIG. 6.—DARK-FIELD CONDENSER

aperture of the oil-immersion lens. It is placed in the objective by unscrewing the lower end.
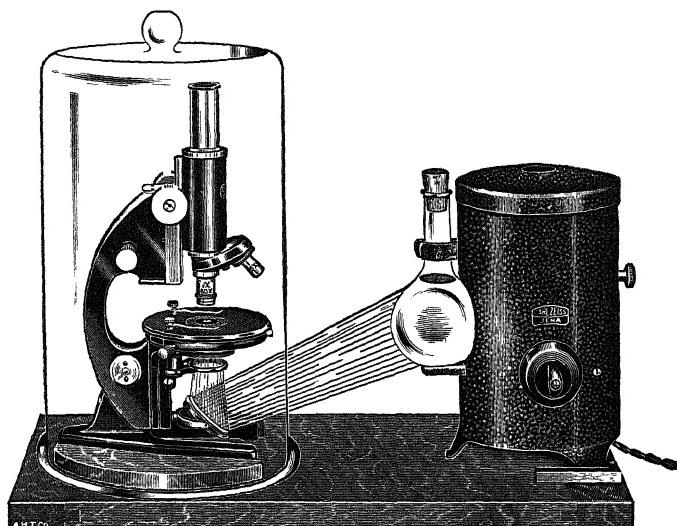
FIG 7.—ZEISS DARK-FIELD ASSEMBLY.

It is recommended to have a microscope with these accessories solely for dark-field work (Fig. 7). It should be fitted with the special condenser which is left in place; also with an oil-immersion lens with the funnel stop in posi-

tion. One or two eyepieces are required; also a very strong light (see Fig. 7). Such equipment is inexpensive and a great time-saver; it also means more satisfactory work in the end because of better centering of light by leaving the special condenser in place.

9. Good artificial light is a practical necessity and a great convenience at all times. Almost any strong light which is diffused through a frosted globe will give fair results (Fig. 8).

A blue glass disk is supplied with the microscope to be placed in a supporting ring beneath the condenser for the purpose of counteracting the yellow rays. Manufacturers have now made available a number of excellent lamps which, when used with nitrogen-filled tungsten lamp and "daylight-glass," transmit a light practically identical with daylight.

The small lamp shown in Figure 9 is very serviceable and inexpensive. It can be placed directly under the stage of the microscope if the mirror is removed.

FIG 8.—MICRO LAMP, CHALET FORM

For dark-field microscopy especially strong light is required, usually in the nature of an arc lamp (Fig. 10). Two kinds are available: that fed by hand and that with an automatic feed (more expensive but excellent for prolonged examinations).
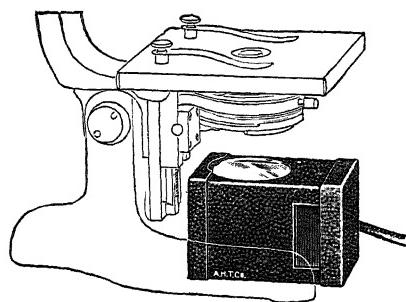
FIG. 9.—MICRO LAMP, SUBSTAGE FORM.
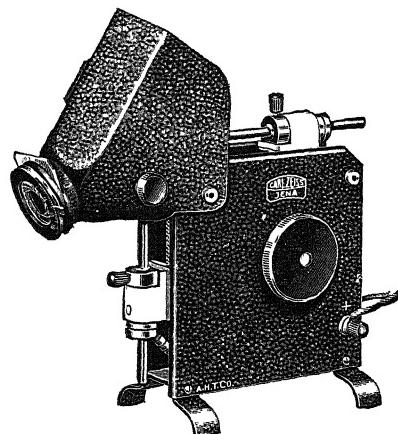
FIG 10.—ELECTRIC ARC LAMP (AUTOMATIC FEED).

10. A good micrometer is a practical necessity, as the importance of size in identification of microscopic structures cannot be too strongly emphasized. Even very rough measurements will often prevent humiliating blunders. The

principal microscopic objects which are measured clinically are bacteria, animal parasites and their ova, and blood corpuscles. The metric system is used almost exclusively. For very small objects, 0.001 millimeter has been adopted as the unit of measurement, under the name *micron*. It is represented by the Greek letter μ. For larger objects, where exact measurement is not essential, the diameter of a red blood corpuscle (7 to 8 micra) is sometimes taken as a unit. Of the several methods of measurement, the most convenient and accurate is the use of a micrometer eyepiece (Fig. 11). In its simplest form this is similar to an ordinary eyepiece, but it has within it a glass disk upon which is ruled a graduated scale. When this eyepiece is placed in the tube of the microscope, the ruled lines appear in the microscopic field, and the size of an object is readily determined in *terms of the divisions of this scale*. The value of these divisions in millimeters manifestly varies with different magnifications. Their value must, therefore, be determined separately for each objective. This is accomplished through use of a stage micrometer—a glass slide with carefully ruled scale divided into subdivisions, usually hundredths of a millimeter.
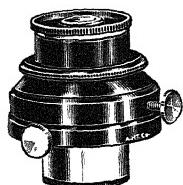
FIG 11.—MICROMETER EYEPIECE WITH MOVABLE SCALE
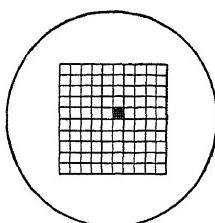
FIG. 12.—OCULAR MICROMETER DISK.

The stage micrometer is placed upon the stage of the microscope and brought into focus. The tube of the microscope is then pushed in or pulled out until two lines of the one scale exactly coincide with two lines of the other. From the number of divisions of the eyepiece scale, which then correspond to each division of the stage micrometer, the value of the former in micra or in fractions of a millimeter is easily calculated. *This value, of course, holds good only for the objective and the tube length with which it was found.* The counting side of the hemacytometer will answer in place of a stage micrometer, the lines which form the sides of the small squares used in counting red blood corpuscles being 50 micra apart. When using the counting chamber with an oil-immersion lens a cover must be used, otherwise the oil will fill the ruled lines and cause them to disappear. Any eyepiece can be converted into a micrometer eyepiece by placing a micrometer disk—a small circular glass plate with ruled scale—ruled side down upon its diaphragm (Fig. 12). If the lines upon this are at all hazy the disk has probably been inserted upside down or else the diaphragm is out of its proper position. Usually it can be pushed up or down as required.

11. A useful *pointer* can be made by placing a straight piece of hair across the opening of the diaphragm of the eyepiece, cementing one end with a tiny drop of balsam, and cutting the hair in two in the middle with small scissors. When the eyepiece is in place, the hair appears as a black line extending from the periphery to the center of the microscopic field. If the pointer does not

appear sharply defined it is out of focus, and the diaphragm must be raised or lowered a little within the ocular.

12. For certain purposes such as platelet counting, reticulocyte counts, etc., it is sometimes advisable to reduce the size of the microscope fields. Any of the following methods can be recommended:

(a) Cut a circular piece of paper which will fit into the eyepiece; then cut a small square in the center of the paper. Remove the top lens of the eyepiece, allow the paper to rest on the diaphragm or shelf and replace the lens.

(b) Glue or cement four pieces of hair across the diaphragm of the eyepiece in such a way that they will form a small square.

(c) With a diamond, scratch two lines on a cover glass so that they will intersect and form four squares. Glue or cement the cover glass to the paper and insert in the eyepiece.

13. The vernier of the mechanical stage cannot be relied upon to mark a particular field upon a permanent preparation because it is impossible to replace the stage in exactly the same position after it has been removed, and because its position is frequently changed by the slight knocks which it receives. There are on the market several "object markers" by which a desired field can be marked with ink, or by a circle scratched on the cover glass by a minute diamond, while the slide is in place on the microscope. The circle is easily located with a low power. In the absence of these one can, while using the low power, place minute spots with a fine pen at the edge of the field on opposite sides.

USE AND CARE OF MICROSCOPE

1. For the benefit of beginners it may be advisable first to point out the essential parts (see Fig. 1).

2. Objectives are "corrected" for use under certain fixed conditions, and they will give the best results only when used under the conditions for which corrected. The most *important corrections* are: (a) for tube length, (b) for thickness of cover glass, and (c) for the medium between objective and cover glass.

The tube length with which an objective is to be used is usually engraved upon it—in most cases it is 160 millimeters. The draw tube of the microscope should be pulled out until the proper length (measured with ocular and objective removed) is obtained. The length is indicated by the graduations upon the side of the draw tube, but in some cases this scale is made for use without a nosepiece. When a nosepiece is attached it adds about 15 millimeters to the tube length.

The average No. 2 cover glass is about the thickness for which most objectives are corrected—usually 0.17 or 0.18 millimeter. One can get about the right thickness by buying No. 2 covers and discarding the thick ones, or by buying No. 1 covers and discarding the thinner ones. Slight differences in cover-glass thickness can be compensated by increasing the length of tube

when the cover is too thin, and decreasing it when the cover is too thick. This should be done with a spiral motion while supporting the body tube with the other hand. The amount of correction necessary will depend upon the focal length and numeric aperture of the objective. With a 4 millimeter objective of 0.85 numeric aperture a difference of 0.03 millimeter in cover-glass thickness requires a change of 30 millimeters in the tube length. Many high-grade objectives are supplied with a "correction collar," which accomplishes the same end. While for critical work, especially with apochromatics, cover-glass thickness is very important, one pays little attention to it in the clinical laboratory. A high-power dry lens always requires a cover, but its exact thickness is unimportant in routine work. Very low-power and oil-immersion objectives may be used without any cover glass.

3. The correction for the medium between objective and cover glass is very important. This medium may be either air or some fluid, and the objective is hence either a "dry" or an "immersion" objective. The immersion fluid generally used is an especially prepared cedar oil, which gives great optical advantages because its index of refraction is the same as that of cover glass. It is obvious that only objectives with very short working distance, as the 2 millimeter, can be used with an immersion fluid.

4. *To use an oil-immersion objective* a suitable field for study should first be found with the low power. A drop of immersion oil is placed on the slide and the objective lowered until it is in contact with the oil and almost touches the slide. For the best work a drop of oil should also be placed between the condenser and the slide. With the eye looking into the microscope, the objective is very slowly raised until the objects on the slide are in focus. In order to avoid air bubbles the oil must be placed on the slide carefully and without stirring it. Bubbles are a frequent source of trouble, and should always be looked for when an immersion objective does poor work. They are readily seen by removing the eyepiece and looking down the tube. If they are present, the oil must be removed and a new drop applied. Immediately after use both objectives and slide should be wiped clean with lens paper or a soft linen handkerchief. In an emergency, glycerin may be used instead of cedar oil, but, of course, with inferior results.

5. After one has arranged the microscope in proper relation to the source of light, whether this be daylight or any of the artificial sources mentioned above, the next step is to secure an evenly illuminated field of view without mottling or any trace of shadows. This is accomplished by manipulating the mirror and the condenser.

Illumination may be either central or oblique, depending upon the direction in which the light enters the microscope. To obtain *central illumination* the mirror should be so adjusted that the light from the source selected is reflected directly up the tube of the microscope. This is easily done by removing the eyepiece and looking down the tube while adjusting the mirror. The eyepiece

is then replaced, and the light reduced as much as desired by means of the diaphragm.

Oblique illumination is obtained in the more simple instruments by swinging the mirror to one side, so that the light enters the microscope obliquely. The more complicated instruments obtain it by means of a rack and pinion, which moves the diaphragm laterally. Beginners frequently use oblique illumination without recognizing it, and are thereby much confused. If the light be oblique, an object in the center of the field will appear to sway from side to side when the fine adjustment is turned back and forth.

6. The *amount of light* admitted is also important. It is regulated by the diaphragm.

The bulk of routine work is done with central illumination, and therefore every examination should begin with it. Each of the forms of illumination, however—central and oblique, subdued and strong—has its special uses and demands some consideration. The well-known rule, "Use the least light which will show the object well," is good, but as stated by Todd and Sanford, it does not go far enough.

In studying any microscopic structure one considers (*a*) its color, (*b*) its outline, and (*c*) its surface contour. No one form of illumination shows all of these to the best advantage. It may, therefore, be necessary to change the illumination many times during a microscopic examination.

(*a*) *To see color best, use central illumination with strong light.* The principle is that by which a stained-glass window shows the purest color when the light is streaming through it. Strong central light is, therefore, to be used for structures such as stained bacteria, whose recognition depends chiefly upon their color, and alternating with other forms, for stained structures in general.

(*b*) *To study the outline of an object, use very subdued central illumination.* The diaphragm is closed to the point which trial shows to be best in each case. This illumination is required by delicate colorless objects, such as hyaline tube casts and cholesterol crystals, which are recognized chiefly by their outline. The usual mistake of beginners is to work with the diaphragm too wide open. Strong light will often render semitransparent structures entirely invisible.

(*c*) *To study surface contour, use oblique light of a strength suited to the color or opacity of the object.* In routine work oblique illumination is resorted to only to study more fully some object which has been found with central illumination, as, for instance, to demonstrate the cylindric shape of hyaline tube casts.

7. The condenser is constructed for parallel rays of light. With daylight, therefore, the plane mirror should be used, while for the divergent rays of ordinary artificial light the concave mirror, which tends to bring the rays together, is best.

It is very important that the condenser be accurately centered in the optical

axis of the instrument, and most high-grade instruments have centering screws by which it can be adjusted at any time. The simplest way to recognize whether the condenser is centered is to close the diaphragm beneath it to as small an opening as possible, then remove the eyepiece and look down the tube. If the diaphragm opening does not appear in the center of the field, the condenser is out of center.

8. The degree of magnification should always be expressed in diameters, not times, which is a misleading term. The former refers to increase of diameter; the latter, to increase of area. The comparatively low magnification of 100 diameters is the same as the apparently enormous magnification of 10,000 times.

In practice, magnification can be increased in one of three ways:

(a) *Drawing out the tube.* Since the increased tube length interferes with spheric correction, it should be used only with the knowledge that an imperfect image will result.

(b) *Using a higher power objective.* As a rule, this is the best way, because resolving power is also increased; but it is often undesirable because of the shorter working distance, and because the higher objective often gives greater magnification than is desired, or cuts down the size of the real field to too great an extent.

(c) *Using a higher power eyepiece.* This is the simplest method. It has, however, certain limitations. When too high an eyepiece is used, there results a hazy image in which no structural detail is clearly seen. This is called "empty magnification," and depends upon the fact that the objective has not sufficient resolving power to support the high magnification. It has been aptly compared to the enlargement, by stretching in all directions, of a picture drawn upon a sheet of rubber. No new detail is added no matter how great the enlargement. The extent to which magnification can be satisfactorily increased by eyepiecing depends wholly upon the resolving power of the objective, and consequently upon the numeric aperture (N. A.). The greatest total or combined magnification which will give an *absolutely* crisp picture is found by multiplying the N. A. of an objective by 400. The greatest magnification which can be used at all satisfactorily is 1000 times the N. A. For example: The ordinary 1.9 millimeter objective has an N. A. of 1.30; the greatest magnification which will give an absolutely sharp picture is 520 diameters, which is obtained approximately by using a 5.5× eyepiece. Higher eyepieces can be used, up to a total magnification of 1300 diameters (12.5× eyepiece), beyond which the image becomes wholly unsatisfactory.

9. Optically, it is a matter of indifference whether the instrument is used in the vertical position or inclined. Examination of fluids requires the horizontal stage, and since much of the work of the clinical laboratory is of this nature it is well to accustom oneself to the use of the vertical microscope. While working one should sit as nearly upright as is compatible with comfort, and the height of the seat should be adjusted with this in view (Fig. 13).

10. It is always best to "focus up," which saves annoyance and probable damage to slides and objectives. This is accomplished by bringing the objective nearer the slide than the proper focus, and then, with the eye at the eyepiece, turning the tube up until the object is clearly seen. The fine adjustment should be used only to get an exact focus with the higher power objectives after the instrument is in approximate focus. It should not be turned more than one revolution.

11. There will be less fatigue to the eyes if both are kept open while using

FIG 13.—PROPER POSITION AT THE MICROSCOPE

Both eyes open. Body erect. (From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore)

the microscope, and if no effort is made to see objects which are out of distinct focus. Fine focusing should be done with the fine adjustment; an experienced microscopist keeps his fingers almost constantly upon one or other of the focusing adjustments.

12. It is very desirable that one train himself to work with the low-power objective as much as possible, reserving the higher power for detailed study of the objects which the low power has found. This makes both for speed and for accuracy. A search for tube casts, for example, with the 4 millimeter objective is both time-consuming and liable to failure. Even such minute structures as nucleated red corpuscles in a stained blood film are more quickly

found with an 8 millimeter or even a 16 millimeter objective combined with a high ocular than with the oil-immersion lens. It is difficult for one who has not measured it to realize how small is the "real field," that is, the actual area of the slide which is seen through the microscope.

13. To be seen most clearly, an objective should be brought to the center of the field. *Acuity of vision will be greatly enhanced and fatigue lessened if all light except that which enters through the microscope be excluded from both eyes.* Strong light should not be allowed to fall directly upon the surface of the slide, as this clouds the image, especially with low powers. To this end various eye shades have been devised, and some workers go so far as to work inside a small tent constructed of strips of wood covered with black cloth, the source of illumination being placed outside the tent.

14. Many good workers advise against the use of spring clips to hold the slide against the stage of the microscope. Manipulation of the slide with the fingers alone certainly gives good training in delicacy of touch, and is desirable when examining infectious material which might contaminate the clips, or when one must detect slight pressure of the objective upon the cover glass, as in studying a hanging-drop preparation. For the majority of examinations, however, it is more satisfactory to use a clip at one end of the slide, with just sufficient pressure to hold the slide without interfering with its freedom of movement.

15. The microscope should be kept scrupulously clean, and dust must not be allowed to settle upon it. *When not in use the instrument should be kept in its case or under a cover.*

16. Lens surfaces which have been exposed to dust only should be cleaned with a camel's-hair brush. A small brush and a booklet of lens paper should always be at hand in the microscope case. Those surfaces which are exposed to finger marks should be cleaned with lens paper, or a soft linen handkerchief, moistened with water if necessary. The rubbing should be done very gently and with a circular motion. Particles of dirt which are seen in the field are upon the slide, the eyepiece, or the condenser. Their location can be determined by moving the slide, rotating the eyepiece, and lowering the condenser. Dirt on the objective cannot be seen as such; it causes a diffuse cloudiness. When the image is hazy, the objective probably needs cleaning; or in case of an oil-immersion lens, there may be bubbles in the oil.

17. Oil and balsam which have dried upon the lenses—an insult from which even dry objectives are not immune—may be removed with xylol; but this solvent must be used sparingly and carefully, as there is danger of softening the cement between the components of the lens. Some manufacturers now claim to use a cement which resists xylol. Care must be taken not to get any alcohol upon the brass parts, as it will remove the lacquer. Balsam and dried oil are best removed from the brass parts with xylol.

18. When the vulcanite stage becomes brown and discolored, the black color can be restored by rubbing well with petroleum.

DARK-FIELD MICROSCOPY

Procedure.—1. Place the funnel stop in the oil-immersion objective.

2. A small drop of the fluid to be examined is placed on a clean slide of the correct thickness and covered with a clean cover glass. The thickness of the slide is important owing to the need of accurately focusing the condenser, and the proper thickness to be used with a particular condenser is generally engraved upon its mounting. This is usually between 1 and 1.55 millimeters. The layer of fluid must be thin. The slide and cover must be free from scratches; air bubbles must be avoided, and also any excess of objects (blood corpuscles, pus cells, etc.) other than those which are sought, since all of these tend to brighten the background and thus reduce contrast.

FIG. 14.—A DARK-FIELD CONDENSER WITH LIGHT ATTACHED.

3. The source of light should be strong artificial light furnished by an arc lamp. A dark-field condenser with light attached is very convenient (see Fig. 14).

4. The regular substage condenser is removed, the dark-field condenser is
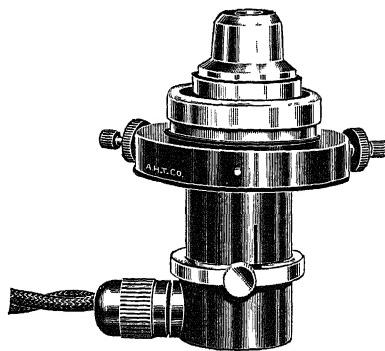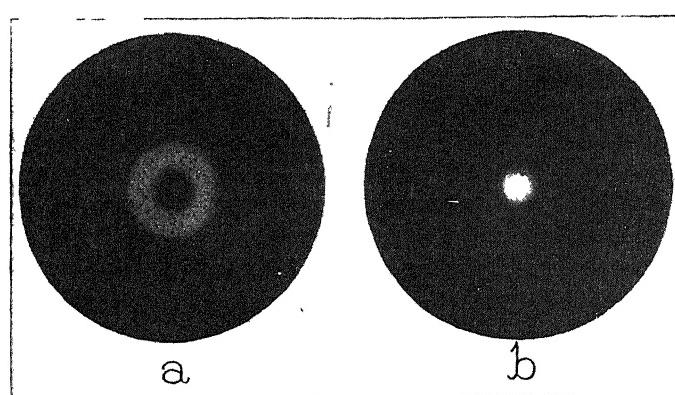
FIG. 15.—DARK-FIELD ILLUMINATION.

(a) Improper illumination because the condenser is above or below the correct focus. (b) Proper illumination with the bright spot when the condenser is correctly focused. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

inserted in its place, and accurately centered in the optic axis. To facilitate centering, a series of concentric circles is generally ruled on the top of the condenser. These circles are brought to the center of the field of a low-power objective by means of centering screws provided for the purpose.

5. A drop of immersion oil is then placed on the apex of the condenser, the slide is placed in position on the stage, and the condenser is raised until the oil is in contact with the under surface of the slide. The low-power objective is now focused on the slide. If the light be sufficiently intense and the mirror properly adjusted, a circle or a spot of light should be seen in the center of the field. A circle indicates that the condenser is decidedly above or below its correct position (Fig. 15). The condenser is then focused by raising or lowering it until the circle becomes a spot of light and this spot becomes as small and as bright as it is possible to make it. This spot is also utilized for centering such condensers as are not provided with the concentric centering circles mentioned above. The low-power objective is finally replaced by the higher power with which the examination is to be made and this is brought to focus and used in the ordinary way.

6. For dark-field work the various adjustments must be much more exact than for bright-field. The most frequent causes of failure to secure a satisfactory dark-field with brilliantly lighted objects which appear to be self-luminous are:

(a) Use of an objective of too high aperture. When the regular oil-immersion objective is used, its aperture must be reduced by means of the stop provided by the makers.

(b) Failure accurately to focus and center the condenser. Very slight readjustments of condenser or mirror after the examination is begun may remedy matters, provided the slide is not too thick to permit accurate focusing.

(c) Inclusion of air bubbles in the preparation or in the oil above or below the slide. It is generally necessary to remove the oil and apply again.

(d) Inclusion of too many microscopic objects in the field. This may be remedied by diluting the fluid to be examined, or by reducing the thickness of the preparation by means of slight pressure on the cover glass.

CHAPTER II

METHODS FOR HOUSIÑG, FEEDING, INOCULATING, BLEEDING AND
AUTOPSYING ANIMALS, AND DIAGNOSIS OF ANIMAL DISEASES

Principles.—1. Suitable animals are indispensable for certain laboratory
procedures, as follows:

(a) For the detection of tubercle bacilli in sputum, urine and other mate-
rials; for the detection of tetanus and other anaerobic bacilli in wound dis-
charges; for the detection of glanders, anthrax, tularemia and other organisms
in various discharges and materials; for the detection of *Spirochaeta pallida* in
spinal fluid and various tissues; for securing a rapid growth of pneumococci
for type differentiation in relation to the serum treatment of pneumonia; for
testing the virulence of diphtheria bacilli in relation to the lifting of quarantine
in diphtheria and for raising the virulence of some organisms, notably strep-
tococci and pneumococci.

(b) For aiding the isolation of certain organisms, as tubercle and glanders
bacilli, pneumococci, etc.

(c) For the preparation of vaccines and the propagation of certain viruses
that cannot be cultivated artificially, as in smallpox and rabies.

(d) For preparing sera for diagnostic immunological reactions like com-
plement, agglutinins, precipitins and various hemolysins.

(e) For testing the curative activity of various chemical agents like
arsphenamine and other organic arsenicals employed in the treatment of syph-
ilis and other diseases.

(f) For the preparation of various immune sera employed in the prophy-
laxis and treatment of diphtheria, tetanus, meningococcus meningitis, gangrene,
pneumonia, etc.

(g) For testing the antibody strength of diphtheria, tetanus, pneumococcus
and other immune sera.

2. Animals should be healthy, selected with care, and provided with com-
fortable and clean housing as well as sufficient and appropriate food.

3. Few of the methods of injection and bleeding produce any more pain
than similar procedures in human beings, but all major operative procedures
should be conducted under full anesthesia and all animals at all times should
be handled and treated with the tender care that their great service to human-
ity, as well as to the lower animals themselves, well merits.

HOUSING ANIMALS

1. The cages for small animals such as rabbits, guinea-pigs, mice and rats should be so constructed and arranged that the animals are kept dry, comfortable and sanitary. Avoid overcrowding. Separate cages should be provided for normal unused animals.

2. Animals from outside sources should be carefully inspected for evidence of dis-
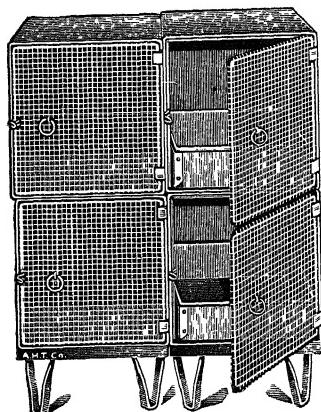
FIG. 16.—ANIMAL CAGE
(Lewis).
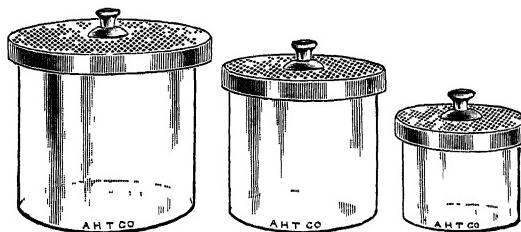
FIG. 17.—ANIMAL JARS FOR MICE, RATS AND
GUINEA-PIGS.

ease before being placed in the normal cages. If space permits, the new animals should be quarantined for a week or ten days.

3. The cages should be constructed to permit thorough cleansing and dis-
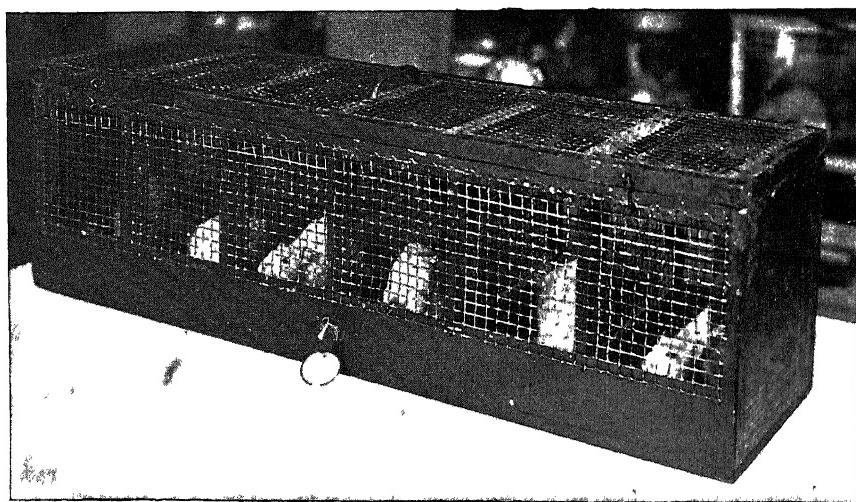
FIG. 18.—A CAGE FOR KEEPING RAT AND MICE SEPARATELY.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

infection and arranged for sufficient light and ventilation. Various models are available from supply houses (Figs. 16 and 17) or may be built to meet special requirements.

4. Special isolated quarters or cages should be provided for animals inoculated with very infectious material and especially that known or suspected of containing *B. anthracis, B. mallei* (*Pfeifferella mallei*), *B. tularense* (*Pasteurella tularensis*), rabies, hog cholera, foot and mouth disease, etc.

5. Inoculated animals should be kept alone in cages to guard them from being annoyed and even injured by normal animals (Fig. 18).

IDENTIFICATION OF ANIMALS

1. **Tagging.**—For rabbits and guinea-pigs a small aluminum tag (Fig. 19) may be used. It is held in place by small staples passed through the eyelets of the tag and then through the ear and the ends bent over.

For larger animals as horses, cows, pigs, etc., there are various types of tags which are supplied with special instruments for attaching. These can be obtained through veterinary supply houses.

2. **Banding.**—This method of identification is chiefly employed for fowl. A band bearing the number is fastened around the leg. There are several types, commonly called "leg bands," which can be purchased from poultry supply houses.

3. **Description.**—If this method of identification is employed alone, animals of different markings or color should always be selected if more than one is to be placed in a cage. Rubber stamp drawings of the mouse, rat, guinea-pig and rabbit may be purchased to facilitate these records.

This method is usually used in conjunction with the tagging method to insure proper identification should the tag be lost.

FIG. 19.—ANIMAL TAGS.

Any deformities or peculiar markings should also be carefully noted.

The sex should be recorded as a part of the description (male ♂ and female ♀).

4. **Marking.**—Small animals like mice and rats may be marked by coloring the hair and skin with dyes (saturated alcoholic solutions of fuchsin or picric acid) on the body or along the tail. Cages should be labeled.
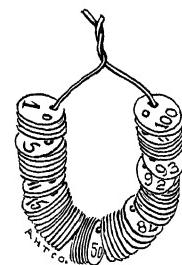
FEEDING AND CARE OF ANIMALS

1. Inoculated animals should be observed at least once or twice a day for symptoms of illness.

2. Dead animals should be removed immediately for autopsy or to a cold room. When removing an animal from its cage, be sure to write a note giving time of death and the cage from which it was removed.

3. When an animal dies after inoculation with infectious material, the cage should be thoroughly disinfected or sterilized.

4. An adequate supply of water should be furnished at all times and in such manner as will avoid soiling of the cage and bedding.

5. All food should be carefully inspected to avoid feeding spoiled materials such as rotten roots, wilted greens, moldy grains, etc.

6. Feed animals once a day and as near the same time as possible. The amount may be judged by what is left over from previous feedings.

7. Do not make any sudden changes in the routine diet. All changes should be gradual. For herbivorous animals, clover or alfalfa hay should be given during the winter months. Greens, such as grass, clover, lettuce, spinach, etc., should be given during the summer months. Salt may be supplied in the form of a salt brick attached to the cage.

8. For *rabbits and guinea-pigs* oats may be kept in the cages at all times. Chopped carrots, beets or cabbage should be a part of the daily ration.

9. *Mice* may be given bread daily and especially soaked in milk. A mixture of several grains should be kept constantly in the cage.

10. *Rats* may be given a mixed diet of cooked and uncooked foods including meats. A cooked mixture of vegetables to which meat scraps and grains are added is recommended, as they do well on a mixed diet similar to that of human beings.

DISEASES OF GUINEA-PIGS

1. **Pneumonia.**—The causative organism in most cases is a hemolytic streptococcus. Occasionally sporadic cases are due to infection with the pneumococcus. The *B bronchisepticus* (*Alcaligenes bronchisepticus*) and Friedlander's bacillus (*Encapsulatus pneumoniae*) have been reported as producing pneumonia in these animals. The symptoms are loss of appetite, roughened coat, and rapid breathing.

Autopsy findings are congestion with consolidation of lungs, which may be associated with pleural and pericardial exudates of a serous or fibrinous character.

2. **Abscesses.**—Abscesses result from an infection of the lymph glands with *hemolytic streptococci*. They often become very large and are usually encapsulated. The contents are a thick and creamy pus from which streptococci can be isolated.

They may become enzoötic or even cause epizoötics among guinea-pigs. This condition is often called "epizoötic lymphadenitis," and the lymph nodes of the neck and axilla are most commonly infected. It is not usually fatal unless the abscesses become so large as to interfere with the function of important organs.

After rupture and drainage the animal usually recovers. It is advisable to incise, remove the pus, and allow good drainage.

3. **Paratyphoid.**—This disease is caused by bacteria belonging to the Salmonella group. The organisms usually encountered are closely related to the *B aertrycke* or the *B. enteritidis.*

The symptoms are loss of appetite, roughened fur, emaciation and weakness. In acute cases death often occurs before symptoms are noted. Out-

breaks among laboratory animals may cause considerable loss. The mortality varies from 4 to 70 per cent.

At autopsy, the spleen and liver show the most constant changes. The former is enlarged and soft, studded with small foci or large yellowish nodules and often covered with a plastic exudate. The liver shows small necrotic foci. The intestines are injected and show swollen Peyer's patches. Pleurisy, pneumonia and purulent endometritis may be present.

4. **Pseudotuberculosis.**—This disease is characterized by the formation of whitish nodules in the liver and spleen associated with enlarged lymph glands which often become abscessed. The causative organism is the *B. pseudotuberculosis (rodentium)*, which is a small, coccoid, gram-negative, nonmotile and distinctly bipolar organism. It will grow on ordinary culture media under aerobic conditions. It is difficult to demonstrate in chronic lesions. It can be definitely identified by an agglutination test with specific serum.

Three clinical types are recognized: (*a*) septicemic type (death in one or two days); (*b*) emaciation and diarrhea (death in three to four weeks), and (*c*) glandular type.

The diagnosis is made by bacteriological examination and the characteristic lesions.

Control measures are not recommended, because of lack of knowledge concerning the epidemiology of the disease.

5. **Tuberculosis.**—Natural infections of guinea-pigs with tubercle bacilli are not common. Normal guinea-pigs can readily contract the disease from tuberculous cage mates or even from animals in the same room.

DISEASES OF RABBITS

1. **Nasal Catarrh (Snuffles).**—This condition may be caused by bacterial infection or by coccidia. When due to bacteria it is spoken of as "snuffles" and when due to coccidia (*Eimeria stiedae*) as "nasal coccidiosis."

The organisms most commonly found are *B. lepisepticus (Pasteurella cuniculicida)* and *B. bronchisepticus (Alcaligenes bronchisepticus)*. The latter when present is usually associated with the former.

The diagnosis of nasal coccidiosis is made by finding the coccidia in the nasal secretions.

The symptoms are sneezing, nasal discharge which is recognized by wetting of the hair around the nasal openings, or the presence of mucus and emaciation. Inflammation of the eyes is often present in cases due to coccidia. In the bacterial type the infection may extend to the lungs and cause pneumonia.

2. **Pneumonia.**—This is usually the result of extension of infection from the upper respiratory tract. The organisms usually found are the *B lepisepticus* and the *B. bronchisepticus (Alcaligenes bronchisepticus)*.

3. **Coccidiosis.**—This disease is caused by coccidia (*Eimeria stiedae* and *Eimeria perforans*), which are among the commonest parasites found in the

rabbit. Although they are found in the intestinal contents of apparently normal rabbits, they are capable of invading the epithelium and causing a mild catarrhal to a severe type of enteritis (intestinal coccidiosis).

They commonly invade the epithelium of the bile ducts, gallbladder and liver. Multiple small white nodules result from their invasion of the liver (hepatic coccidiosis).

The nasal form of the disease has been given above, under nasal catarrh of rabbits (snuffles).

The diagnosis is made by finding the coccidia in association with enteritis or nodules. The organism is readily demonstrated by placing the contents of a liver nodule, bile, or scrapings of intestinal mucosa on a slide and examining the unstained specimen.

The oöcysts appear as oval bodies from 15 to 25 micra in length.

There is no treatment. Preventive measures should consist of frequent cleaning of cages, separating animals, avoiding grouping, raising floor with wire mesh so droppings can pass through, etc.

4. **Ear Mange.**—This is caused by a mite *Dermatocoptes* (*Psoroptes*) *cuniculi*. The disease is characterized by the formation of thick crusts or deposits on the inside of the ear.

The diagnosis is made by examining scrapings from the ear, unstained, under low power, for the mite.

Infected animals should be separated and treated locally. Remove the scabs and apply one of the following:

Mercuric chloride	1 part
Glycerin	100 parts
Ethyl alcohol (50 per cent).............	200 parts

or

Oil of caraway.......................	1 part
Almond oil	10 parts
Ethyl alcohol (90 per cent)............	3 parts

5. **Giardiasis.**—Giardia may be found quite commonly in the small intestines of rabbits. Whether or not they are harmful is questionable.

6. **Parasitic Cysts.**—Cysts of the dog tapeworm (*Taenia pisiformis*) are commonly found in the mesentery of the rabbit. They are spoken of as "bladder worms" and often occur in large numbers and occasionally in the liver.

The general health of the animal is not affected.

DISEASE OF MICE

Mouse Typhoid (Paratyphoid).—This is the commonest infection and is due to bacilli of the Salmonella group, especially *B. aertrycke* and *B. enteritidis*. Spontaneous epizoötics occur, causing considerable loss; the mortality is high, ranging from 34 to 95 per cent.

Susceptibility varies in different breeds and strains of mice and is influenced by diet and environment.

The diagnosis is established by isolating an organism belonging to the Salmonella group from the spleen or other organs.

DISEASES OF RATS

1. **Lung Disease.**—This is a respiratory infection prevalent among albino rats. Young rats are not susceptible. The symptoms are loss of appetite, rhinitis, conjunctivitis and labored breathing; as the disease becomes chronic there is much depression. The mortality is high.

At autopsy the lungs are always affected; unresolved pneumonias, catarrhal bronchitis with bronchiectases, abscesses and pleurisy are common findings.

Various organisms of dissimilar groups have been isolated from these lesions, none of which have been recognized as the cause. The etiology therefore is still undetermined.

2. **Paratyphoid.**—This disease is due to members of the Salmonella group, particularly the *B. enteritidis* type. The symptoms in acute cases are diarrhea and bloody crusts around the eyes.

The lesions found at autopsy are swollen spleen, multiple necrotic foci, enlarged lymph glands and Peyer's patches. Chronic cases may fail to show any lesions. The organisms can be readily isolated from the spleen and liver.

GENERAL DIRECTIONS FOR THE INJECTION OF ANIMALS

1. Select an appropriate size syringe that does not leak upon being tested with water. Nothing is more unsatisfactory than a leaking syringe, for not only may the hand become soiled, but an unknown quantity of inoculum is lost.

2. Remove the plunger from the barrel, and sterilize all the parts by boiling for at least several minutes. An all-glass syringe or a glass barrel and metal plunger are the most satisfactory. The old-fashioned syringe with washers and rubber-tipped plunger should find no place in a laboratory.

3. After cooling, expel the water and load the syringe. This may be done by drawing the fluid directly into the syringe

Fig 20.—Animal Board.

and measuring the dose by its markings or by pipeting the exact dose into a sterile Petri dish or capsule and drawing up in the syringe.

4. The animal should be fastened or held firmly and in an easy position. Everything should be in readiness, so that the injections may be given thoroughly and carefully. Various holders have been described; those shown in Figures 20 and 21 may be recommended.

5. In preparing the inoculum, care should be exercised that no solid
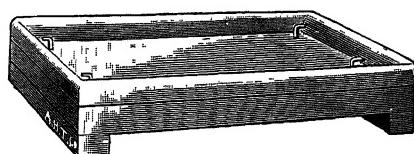
particles enter the syringe. Aside from possibly blocking the needle and inter-fering with the injection, the subcutaneous injection of small fragments may do no particular harm, but in intravenous inoculation they may cause fatal embolism.

6. Air bubbles should be removed. The injection of small bubbles of air into subcutaneous tissues may cause no harm, but when injected into veins they may cause serious disturbances or immediate death. To avoid this, the syringe, after being filled, should be held vertically, with the needle upper-most. The needle should be wrapped in cotton soaked in alcohol and the piston of the syringe pressed upward until all the air is expelled from the barrel and the needle If a drop of inoculum is forced out, it will be collected on the cotton, which should immediately be burned.

7. Injections should be slowly given.

8. When it is necessary to incise the skin in order to reach a vein, an anesthetic may be given. With superficial veins, and in subcutaneous inocu-lations, the injections may be given so readily and easily that no more pain can be felt than that which accompanies similar injections in human beings.

9. When it is necessary to remove the hair from the area to be injected, a small area may be clipped as closely as possible, followed, if necessary, by shav-ing In guinea-pigs the hair may be plucked out; or clip the hair and apply a paste of equal parts of barium sulphide and cornstarch mixed with water. Leave this on for three or four minutes, wash thor-oughly with warm water and dry with a towel This is a particularly good method when large areas of skin are to be prepared. As it may cause irritation, it is well to remove the hair a day in advance of injection.

Fig 21 —Coca-Ramsdell Board

10. Before injection cleanse the skin with 70 per cent alcohol or wipe with 1 :1000 mercurophen or other disinfectant.

INTRACUTANEOUS INJECTION

1. Select white animals or white areas if skin reactions are to be elicited as allergic reactions, diphtheria toxin reactions, etc.

2 Use a 1 c.c syringe with No. 26 needle.

3 Prepare the skin. Pinch up a fold and insert the needle (lumen up) as superficially as possible. A raised, white, anemic spot showing the pits of hair follicles indicates a successful injection. Owing to thin skins of rabbits and guinea-pigs, the injection is by no means easy or simple and requires practice. The amount injected should not exceed 0.1 c.c.

SUBCUTANEOUS INJECTION

1. Injections are usually given in the median line of the abdominal wall or in the groin (Fig. 22).

2. Have the animal held firmly by an assistant or secured to the operating table.

3. Clip the hair where injection is to be made—it is not always necessary to shave the area. Apply a 2 per cent iodine in alcohol solution.

4. Pinch up a fold of skin between the forefinger and the thumb of the

FIG 22—SUBCUTANEOUS INJECTION OF RABBIT.
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co)

left hand; hold the syringe in the right hand, insert the needle into the ridge of skin between the finger and thumb, and push it steadily onward until the needle has been inserted about an inch. Care must be exercised not to enter the peritoneal cavity. Relax the grasp of the left hand and slowly inject the fluid. If the skin is raised, this shows that the injection is subcutaneous. If it is not, the needle should be slightly withdrawn and inserted.

5. Withdraw the needle, and at the same time cover the puncture with a wad of cotton wet with alcohol. A touch of flexible collodion over the puncture completes the operation.

6. If a *solid* inoculum is to be injected, raise a small fold of skin with a pair of forceps, and make a tiny incision through the skin with a pair of sharp-pointed scissors.

7. With a probe, separate the skin from the underlying muscles to form a funnel-shaped pocket.

8. By means of fine-pointed forceps or a glass tube syringe, introduce the inoculum into this pocket and deposit it as far as possible from the point of entrance of the instrument.

9. Close the wound with collodion and cotton. A single stitch with fine thread may be necessary.

INTRAMUSCULAR INJECTION

1. These injections are usually made into the posterior muscles of the thigh or into the lateral thoracic or abdominal muscles.

2. Clip away the hair over the selected area, cleanse, etc., as for subcutaneous injection.

3. Steady the skin over the selected muscles with the slightly separated left forefinger and thumb.

4. Thrust the needle of the syringe quickly into the muscular tissue and slowly inject the fluid.

INTRAVENOUS INJECTION

Rabbit.—1. The posterior auricular vein along the outer margin of the ear is better adapted than a median vein for this purpose.

2. If a number of injections are to be made, commence as near the tip of the ear as possible, as the vein may become occluded with thrombi, and subsequent inoculations may then be given nearer and nearer the root of the ear.

3. The animal should be held firmly, as the slightest movement may result in piercing the vein through and through and require reinsertion of the needle. This is accomplished satisfactorily by placing the rabbit upon the edge of the table and holding it firmly there by grasping the neck and front quarters, the assistant at the same time compressing the root of the ear with the thumb and forefinger.

4. If the hair is long, clip it.

5. The ear is struck gently with the fingers and washed with alcohol and xylol; the friction will render the vein more conspicuous.

6. The ear is grasped at its tip and stretched toward the operator, or the vein may be steadied by rolling the ear gently over the left index finger and holding it between the finger and thumb.

7. The inoculum should be free from solid particles and all the air excluded from the syringe. As a general rule, the amount injected should be as small as possible, and the temperature of the inoculum be near that of the body. If the syringe is filled shortly after sterilization, when it has cooled enough

to be comfortably hot to the touch, the heat will warm the injection fluid and not be hot enough to cause coagulation.

8. Hold the syringe as one would hold a pen, and thrust the point of the needle through the skin and the wall of the vein until it enters the lumen of the vein (Fig. 23).

FIG 23—INTRAVENOUS INJECTION OF RABBIT
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co)

The wooden box shown in Figure 24 is very convenient for holding rabbits for intravenous injection or for bleeding from the ears.

9. Direct the assistant to release the pressure at the root of the ear, and *slowly* inject the inoculum. If the fluid is being forced into the subcutaneous
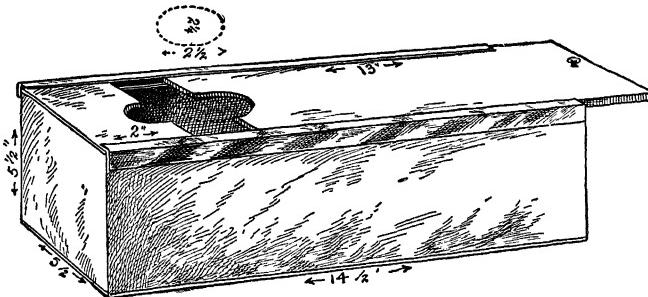
FIG. 24.—A WOODEN BOX FOR RABBITS
(From Kolmer, *Infection, Immunity and Biologic Therapy,* W B Saunders Co.)

tissue, which will be evident at once by the swelling which occurs, the injection must cease and another attempt be made.

10. The needle is quickly withdrawn, a small piece of cotton moistened with alcohol placed upon the puncture wound, and firm compression applied.

Wash the ear thoroughly with alcohol and water to remove xylol, otherwise a low-grade inflammation will follow, which will render subsequent injections more difficult.

Guinea-Pig.—1. The large superficial vein lying on the dorsal and inner aspect of the hind leg of the guinea-pig is well adapted for intravenous injection. Occasionally, however, the vessel may run anteriorly. To use the above described vessel for intravenous administration, a special operating board is required. The board is similar to an ordinary animal board, except that the end to which the hind legs of the animal are tied has a U-shaped piece cut from it. The board is mounted near the center on an extension shaft which is fitted with two joints, the one at the end to which the board is attached being a ball-and-socket joint and the other an adjustable swivel joint. The shaft is

FIG 25 —ROTH'S METHOD FOR INTRAVENOUS INJECTION OF THE GUINEA-PIG.
(From Kolmer, *Chemotherapy*, W. B Saunders Co)

screwed into a metal base which has sufficient weight to hold the board steady when placed in any position. A single operating board, however, may be used, as shown in Figure 25.

2. The procedure for making the injection is as follows: With the board properly placed in a horizontal position, the animal is tied to it securely, abdomen downward, by means of strings. The board is then placed in a vertical position and rotated on its vertical axis slightly so as to bring the dorsal aspect of the right hind leg into view. After clipping the hair from the leg and shaving it, the leg is lifted up slightly by the first or first and second fingers, and the vein dilated by suitable compression. The vessel can now usually be seen through the skin. A small incision, usually about one-fourth of an inch long, is made across the leg from the outer lower to the

upper and inner aspect, but a trifle to the left of the vessel. The subcutaneous tissue is then pushed aside with fine-pointed forceps, thereby permitting the vessel to come into view.

3. The vessel is then entered directly or in the same manner as has been described for the rat—that is, by passing the needle of the tuberculin syringe through the fascia and muscles to the left of the vessel and then entering the vessel from the side. The vessel when dilated permits the ready entrance of a gauge No. 23 needle. However, the needle usually employed is a gauge No. 26, five-eighths of an inch in length. The needle is always introduced well into the lumen of the vein. If entrance into the vessel is direct, subsequent hemorrhage may be controlled readily by pinching it with a small forceps.

4. Injections may be also given in one of the external jugular veins as follows:

(a) A small roll is placed under the neck of the animal to render the operative area tenser and more easily accessible.

(b) A few drops of ether may be given by an assistant, although one soon learns to expose the vein quickly and there is practically no pain after the skin has been incised.

(c) Assistant is directed to hold the head backward in the median line.

(d) Pick up the skin just above and in the middle of the space between the shoulder and the tip of the upper end of the sternum—just above and about in the center of the area where a clavicle in the human would be situated. With small sharp scissors incise the skin for about one-half inch. Separate the subcutaneous tissue gently with forceps; a large vein at once comes into view. Gently dissect it free for about one-quarter inch.

(e) Pick up the vein with a pair of fine forceps, insert the needle of the syringe gently in the long axis of the vein, and slowly inject the fluid (Fig 26).

(f) Withdraw the needle and apply firm pressure with a wad of clean gauze or cotton It is not necessary to tie off the vein. A stitch may be inserted to close the skin wound and flexible collodion applied.

White Mouse.—1. The lateral veins of the tail of the white mouse have been found best suited for intravenous administration purposes. The tail must be free from localized or generalized thickening of the epidermis so as to permit the ready entrance of a gauge No. 23 needle. The use of a rather long needle, 1 inch in length, is essential; it does not bend easily and therefore can be directed forward more readily than a smaller one.

2. A mouse weighing between 15 and 20 grams practically always possesses a soft, pliable tail which can be used without any preparation. When a mouse weighing over 20 grams is used, the lateral veins of the tail are usually covered with rather dense tissue, which precludes their use unless the tail is immersed for about a half minute in warm water (about 50° C.). This procedure both softens the skin and dilates the underlying vessels so that the latter may be successfully used.

3. For holding the mouse, a small tin mailing tube attached to an iron

stand is employed (Fig. 27). One end of the metal mailing tube is fitted with a cork having at the circumference a V-shaped opening, which will admit the tail. The other end of the tube contains several small openings for the purpose of admitting air.

4. The mouse is grasped by the tail with the thumb and forefinger of the left hand and placed in the above-described metal mailing tube, and the cork is inserted so that the tail protrudes through the V-shaped opening. The
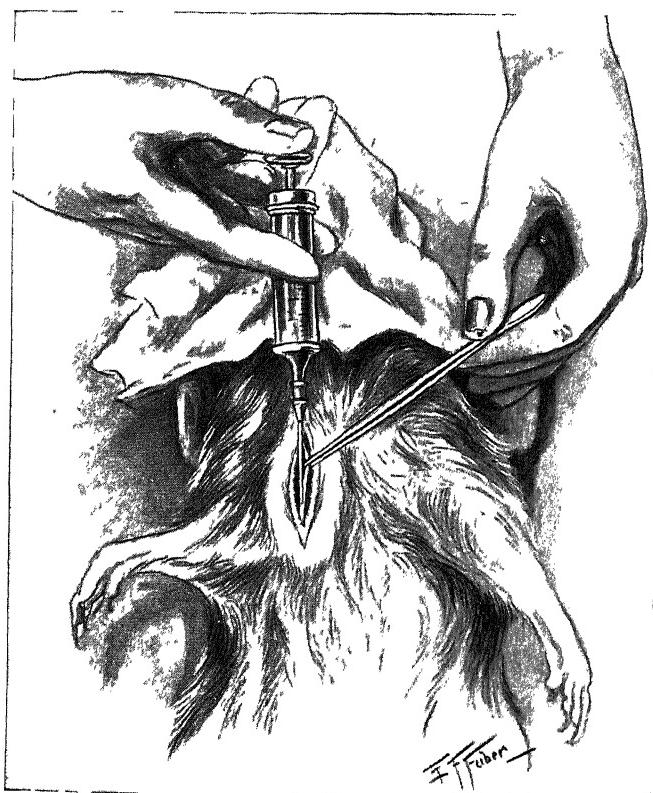
FIG 26—INTRAVENOUS INJECTION OF GUINEA-PIG
(From Kolmer, *Infection, Immunity and Biologic Therapy,* W B Saunders Co)

tail is now straightened by gentle but firm traction and without twisting. The dorsal vein should then appear above, and each lateral to the left and right, respectively.

5. The syringe, usually a 1 c.c., all-glass, tuberculin type, graduated to o o1 c.c., is balanced between the first and middle fingers of the right hand, the hand resting on the little finger; the thumb is thus free to operate the piston of the syringe. With the syringe held nearly parallel to the tail, the needle is pushed through the skin over one of the lateral veins (usually the

left) and then anteriorly and downward into the vein. If an entrance into the vessel is not effected, either raising or lowering the point of the needle while advancing it further will usually succeed in locating the lumen of the vessel.

White Rat.—1. The animal is tied securely by the legs, back downward, to a flat operating board, by means of strings long enough to permit the hind legs to be lifted easily.

2. At the end of the board to which the head is tied are two glass pegs about 1 inch long set in at an angle in order to hold the string which is
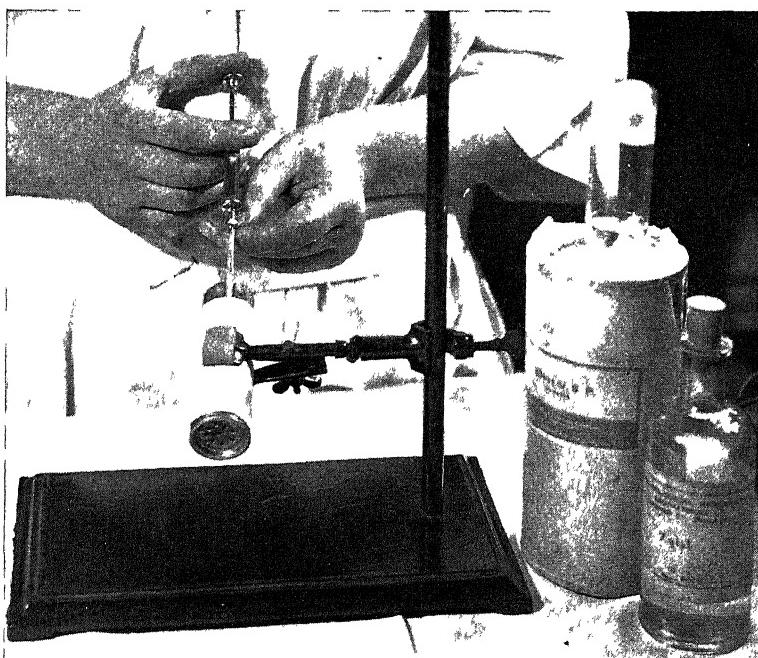
FIG 27—ROTH'S METHOD FOR INTRAVENOUS INJECTION OF THE MOUSE
(From Kolmer, *Chemotherapy*, W. B Saunders Co)

looped over the front legs of the animal. Nails in the other end of the board receive the strings which are looped to the hind legs (Fig. 28).

3. After shaving the hair over the skin area covering the left saphenous vein, the left foot is grasped between the third and middle fingers of the left hand, and an incision about one-fourth to one-half of an inch long is made about one-fourth of an inch to the left of and parallel to the vein. The skin is then rolled over to the right with the first finger of the left hand by drawing the skin on the back of the leg to the left. This will bring the vessel into view. An assistant then makes compression to dilate the vessel. If a syringe is used, it is preferable to employ a 1 c.c., all-glass tuberculin type, graduated to 0.01 c c. and fitted with a gauge No. 26 needle, five-eighths of an inch

in length. The syringe is balanced between the first and middle fingers of the right hand, the hand resting on the little finger; the thumb is thus free to operate the piston of the syringe. The needle is then passed through the fascia and upper surfaces of the muscles, about one-eighth of an inch to the left of the vein and almost parallel to it. Advancing the needle slightly farther, the direction is changed so that the needle will enter the vein from the side. After the injection is made, the skin which was pulled to the right to permit the vessel to come into view, is released and this skin flap and the muscles act as effective mechanical checks to hemorrhage, which is quite profuse if the needle is inserted directly into the vein.

4. If a buret is employed and the injection made by gravity instead of by means of a syringe, a flexible rubber tube is attached to the buret, while the other end of the tube carries a glass tube which is drawn out and ground
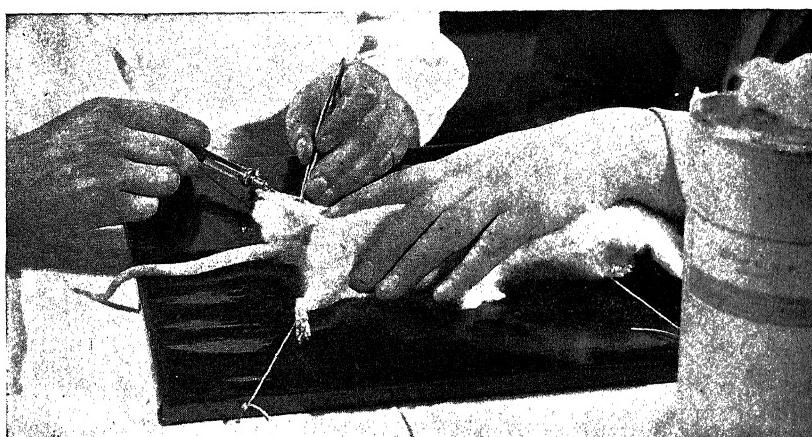
FIG. 28.—INTRAVENOUS INJECTION OF THE WHITE RAT EMPLOYING THE FEMORAL VEIN.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

to fit a gauge No. 25 needle 1 inch long. The glass tube is handled in the same way as the syringe, and the vessel is entered in the same manner as described under the syringe method (Fig. 29).

5. An *external jugular vein* may be used instead and has the advantage of being larger.

The animal is tied to the operating board in the same manner and a wad of cotton placed under the shoulder. The assistant holds the head backwards and to one side with a piece of cotton rendering the tissues of the operative area tense and firm (Fig. 30).

The skin is touched with alcohol and a small incision made just above and about in the center of the area where a clavicle in the human would be situated. The subcutaneous tissues are gently dissected and the vein exposed, which becomes very prominent with respiratory movements. It is well not to attempt entering the vein until it is thoroughly exposed, as otherwise one
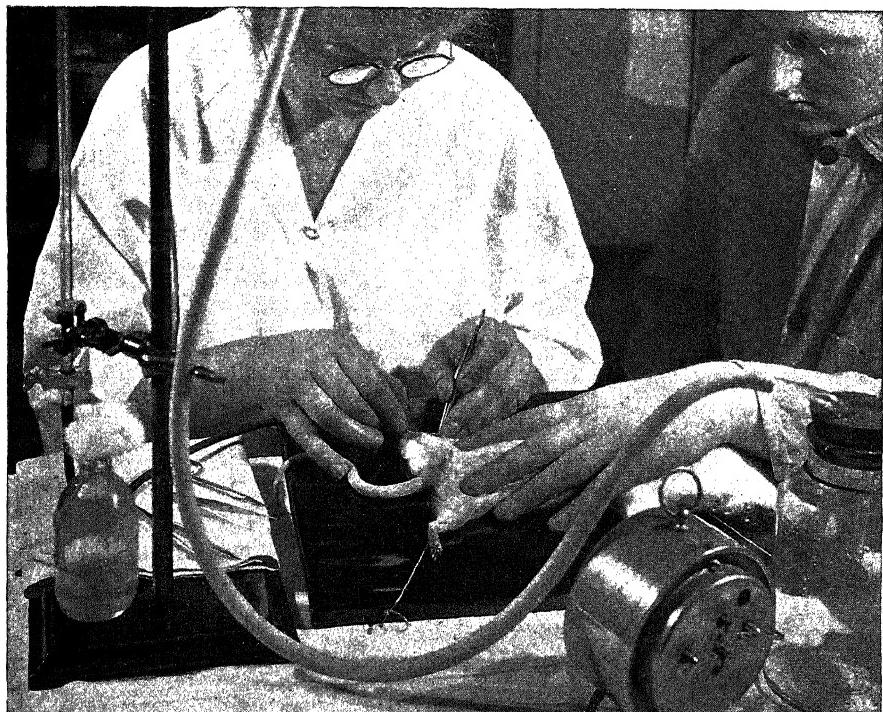
FIG. 29.—INTRAVENOUS INJECTION OF THE WHITE RAT WITH GRAVITY APPARATUS.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)
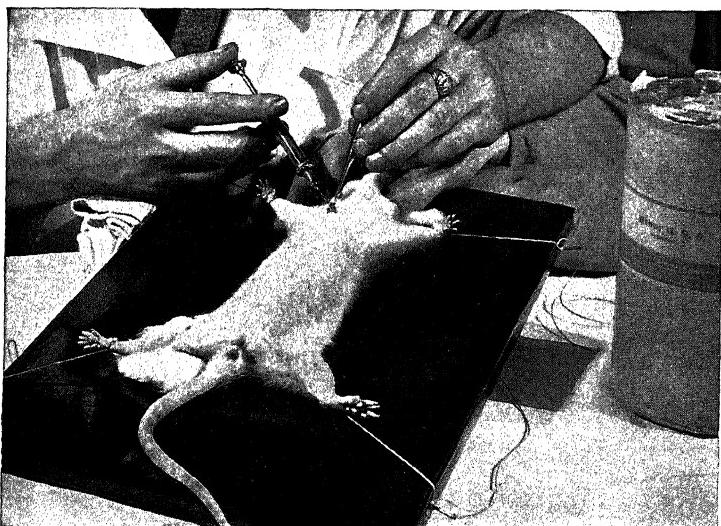
FIG. 30.—INTRAVENOUS INJECTION OF RAT EMPLOYING THE EXTERNAL JUGULAR VEIN.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

may infiltrate the tissues over and about the vein by failure to enter it properly. As soon as the injection has been given the animal is quickly released and the wound requires no attention as infection practically never occurs and healing is rapid.

Dog.—1. Dogs may be injected through the external jugular or popliteal veins. The animal should be fastened to the operating table (Fig. 31).

2. There is a small vein just beneath the skin in the median line, along the anterior surface of the leg, which is readily accessible. Clip away the hair and disinfect with iodine and alcohol. Direct the assistant to grasp the thigh just above the knee to distend the vein and prevent movement, and make a small incision directly in the median line. A small vein is seen at once. Dissect free or pick up gently with fine forceps and insert a small sharp needle. The injection can thus be readily given. Withdraw the needle, apply firm pressure, and insert a single stitch. Bind the wound with a few turns of a gauze bandage or seal with collodion and cotton.

INTRACARDIAL INJECTION

1. Guinea-pigs may be injected by the intracardial route instead of intravenously. The technic is not, as a rule, more difficult, and no ill effects are noticed. Not infrequently, however, attempts to inject in the heart fail, and frequent trials are not permissible on account of the danger of injuring the organ.

2. The animal is tied to the operating board, or held firmly by an assistant; an anesthetic may be given.

3. Determine the point of maximum pulsation to the left of the sternum by palpation, and quickly insert a thin, sharp needle at the selected area. A flow of blood indicates that the needle has entered the heart. Attach the previously filled syringe and slowly inject the contents.

4. Detach the syringe in order to make sure that the injection was intracardial as intended, which is indicated by a flow of blood; then quickly withdraw the needle. The puncture wound may be sealed with collodion.
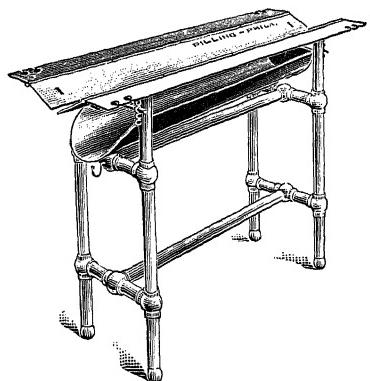
FIG. 31.—DOG OPERATING TABLE.

INTRAPERITONEAL INJECTION

Rabbit.—1. Clip the hair and shave an area about 2 inches in diameter in the median abdominal line just below the umbilicus. Apply 2 per cent iodine in alcohol.

2. Direct an assistant to hold the animal firmly, head down. With the animal in this position the loops of intestine tend to sink toward the diaphragm,
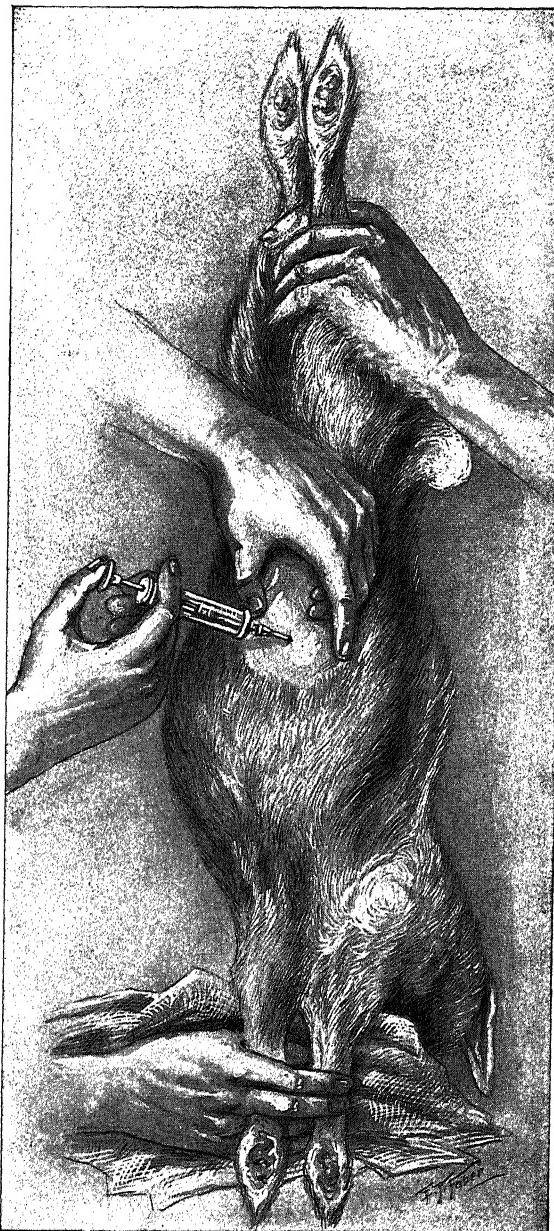
FIG. 32.—INTRAPERITONEAL INJECTION OF RABBIT.

(From Kolmer, *Infection, Immunity and Biologic Therapy,* W. B. Saunders Co.)

leaving an area above the bladder which is sometimes free of intestines (Fig. 32).

3. The syringe is grasped firmly and the needle inserted beneath the skin

for a short distance in the direction of the head in the long axis of the animal, when the hand is raised and the needle forced forward through the peritoneum. When the peritoneum has been entered this is evidenced by a relaxation of the abdominal muscles. The needle is then withdrawn slightly and the injection made.

Guinea-Pig.—1. Direct an assistant to hold the animal firmly upon its back. This is better than fastening it to an operating table, for it permits relaxation of the abdominal wall when the injection is to be made.

2. Pluck the hair in the median abdominal line. A small area may be shaved, although this is not necessary. Disinfect with an application of iodine in alcohol

3. With the left forefinger and thumb pinch up the entire thickness of
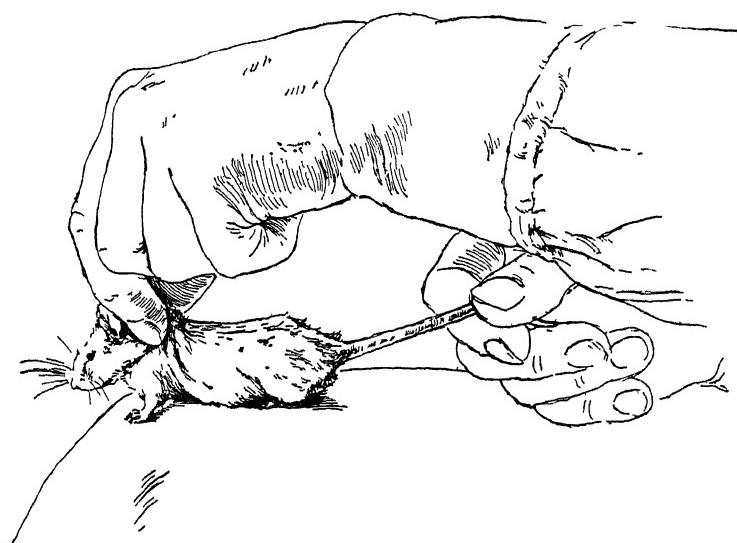
FIG 33.—MANNER OF GRASPING A MOUSE.
(From Wadsworth, *Standard Methods,* Waverly Press, Inc, Baltimore)

the abdominal parietes in a triangular fold, and slip the peritoneal surfaces over each other to ascertain that no coils of intestine are included.

4. Grasp the syringe in the right hand and insert the needle into the fold near its base.

5. Release the fold and inject the fluid. If a swelling forms, this shows that the needle is in the subcutaneous tissues, and another attempt should be made to enter the peritoneum.

6. It may be difficult to pinch up the parietes without including the intestine. In such case straighten out the animal and stretch the skin between the left forefinger and thumb. Insert the needle obliquely until it is beneath the skin. A slight thrust suffices to pierce the peritoneum, when the abdomi-

nal muscles will be felt to relax. Withdraw the needle slightly and inject the fluid.

7. Seal the wound with a touch of collodion.

Mouse.—The technic is practically the same (Figs. 33 and 34).

SUBDURAL INJECTION

Rabbit.—1. Use a No. 18 gauge needle which has been cut off and sharpened to about three-sixteenths of an inch in length, and a 1 c.c. syringe. Sterilize by boiling and fill with inoculum.

2. Shave or clip the hair over the site of injection, which is located a few centimeters posterior to and on a line with the outer canthus of the eye. In this region a small horizontal groove can be detected by feeling with the finger nail. The bone at this point is thin.
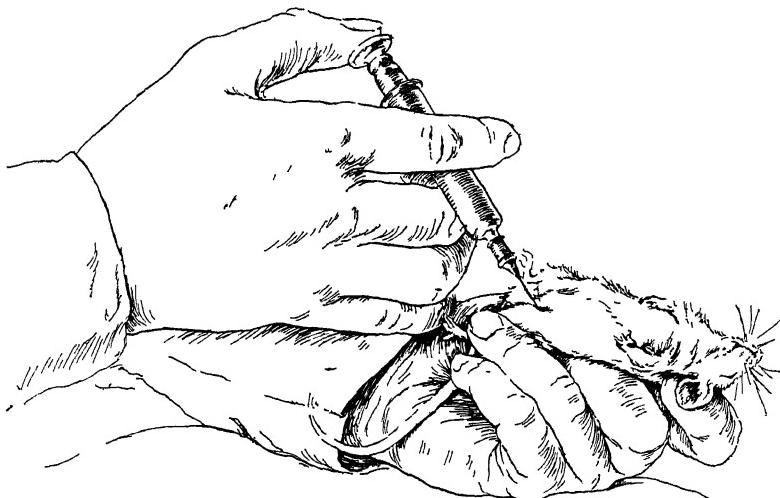
FIG 34.—INTRAPERITONEAL INJECTION OF MOUSE
(From Wadsworth, *Standard Methods,* Waverly Press, Inc., Baltimore.)

3. Place the needle in the groove and force it through the bone into the cranial cavity.

4. Inject the material slowly.

TESTICULAR INJECTION

Rabbit.—1. Sponge the scrotum with tincture of iodine.

2. Fill sterile syringe with material to be inoculated. Use a No. 22 gauge needle about five-eighths of an inch in length.

3. Insert the needle through the skin of the scrotum into the substance of the testicle.

4. Inject the material.

METHODS FOR OBTAINING BLOOD FROM ANIMALS

Rabbit.—FROM THE EAR VEINS.—1. Flip an ear vigorously with the hand, and rub with xylol and alcohol. The xylol produces marked congestion and afterwards should be carefully removed with alcohol and water, as it produces a low-grade inflammatory reaction.
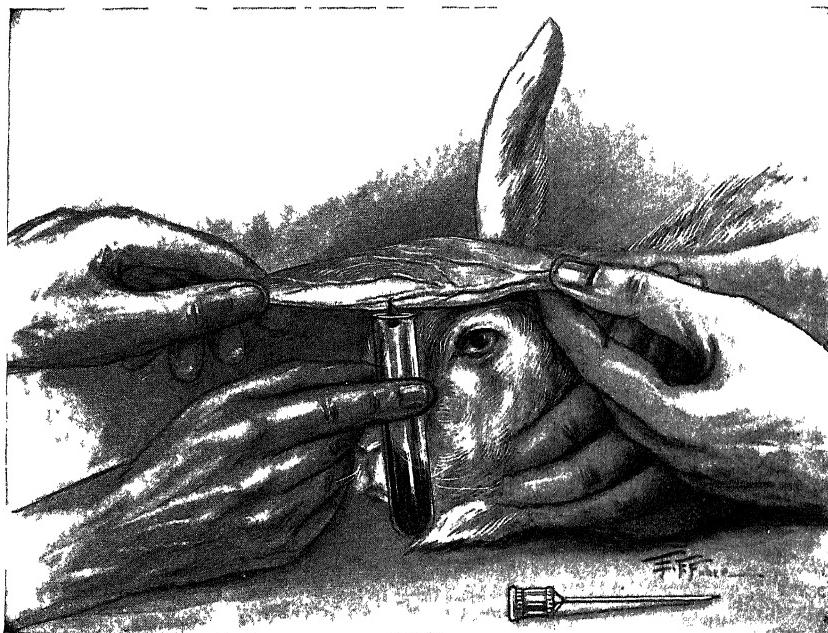
FIG 35 —METHOD OF BLEEDING A RABBIT FROM THE EAR
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W B Saunders Co)

2. Puncture a marginal vein with a large needle. The blood will flow quickly in drops and practically any amount up to 10 c c. or even more may be collected in a centrifuge or test tube (Fig. 35). For making preliminary tests of serum during immunization, 2 c.c. of blood are usually sufficient. Bleeding may be checked by making firm pressure over the puncture.

Another good method is to place the animal in the box shown in Figure 24, turning it up on end so that the animal's head is down (Fig. 36). This permits one to bleed the animal without assistance.

FROM THE HEART.—1. Tie the animal securely to a board and clip the hair from an area of the chest about one and one-half inches in diameter.

2. Determine the point of maximum pulsation and disinfect with tincture of iodine.

3. If 5 to 20 c.c. of blood are required, use a sterile syringe fitted with a No. 17 to 19 gauge needle   If more than 20 c c are required or the animal is to be "bled out," use a 200 c c. bottle with a rubber stopper fitted with

two pieces of glass tubing, one connected with rubber tubing for suction and the other connected by means of heavy-walled rubber tubing with the needle. Sterilize in an autoclave.

4. Etherize lightly unless the worker is experienced, when ether may be omitted as it may cause more discomfort than the puncture.

5. Enter the needle at the point of maximum pulsation, applying gentle suction.

6. Approximately 20 c c. of blood can be taken from a 2000 gram rabbit at intervals of two or three weeks.

FROM THE NECK.—1. Clip the hair from the neck and disinfect with 70 per cent alcohol or 1 per cent lysol solution; dry with a towel

2. Etherize the animal lightly (do not use chloroform).

3 While an assistant holds the animal head down, rapidly sever the neck on both sides with a razor or sharp scissors (avoiding the trachea and esophagus), and collect the blood by a funnel into centrifuge tubes or a dish. Or the following may be used By means of a sterile knife the skin is cut longitudinally and the neck muscles exposed for a consider- able distance.

4. The animal is then held upright by the assistant over a sterile dish or a large sterile funnel, emptying into a cylinder or 50 c.c. centrifuge tube.

FIG 36—METHOD OF BLEEDING A RABBIT FROM THE EAR

(From Wadsworth, *Standard Methods,* Waverly Press, Inc, Baltimore)

5. The operator stretches the neck by carrying the head backward, and severs the large vessels on one or both sides of the neck with a sharp sterile scalpel or razor, avoiding opening the trachea and esophagus.

6. After bleeding, the dish is covered or the tube plugged and set aside for the serum to separate.

Guinea-Pig.—The animal is anesthetized with ether or stunned with a sharp blow and the large vessels of the neck on one side are exposed by a longitudinal incision These are severed, and the blood is collected in a Petri dish or in a centrifuge tube by means of a funnel. Or by means of a sharp-pointed scissors the vessels on one or both sides of the neck may be

incised transversely at one cut, inserting the blade deeply and close to, but avoiding, the trachea and esophagus.

Blood may be obtained by aspiration from the heart of the living animal. A syringe fitted with a No. 20 or 22 needle is employed. The animal is fastened to a board or held by an assistant and lightly anesthetized The point of maximum pulsation is determined and the needle slowly entered into the right chambers of the heart.

As a general rule, 5 to 15 c.c. of blood may be obtained by gentle suction, the amount depending upon the size of the animal. Large male animals (700 grams or more) are recommended and may be used every two to three weeks.

FIG. 37.—METHOD OF SECURING BLOOD FROM THE HEART OF A GUINEA-PIG.
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W B Saunders Co.)

After withdrawal of the needle the animal rapidly recovers, although occasionally bleeding may follow into the pericardial sac (Fig 37).

Sheep.—Blood may easily be obtained from a freshly killed animal. The first flow of blood is discarded, and a portion of the remainder is collected in a large, sterile, thick-walled flask containing glass beads. By shaking vigorously the blood is defibrinated if one desires to obtain corpuscles, or the blood may be collected in a cylinder and defibrinated by whipping with glass rods.

It is usual, however, in large laboratories to keep a sheep and to remove the blood as it may be required. Small amounts may be obtained from the ear vein, larger quantities being secured from an external jugular vein in the following manner:

1. One may do the bleeding alone, although the aid of an assistant is usually necessary, especially if the animal is large and vicious.

2. The sheep is thrown on its back, and the head is held on the knees of an assistant seated on a low box or stool.

3. The operator may straddle the animal to hold down the fore feet, although this is not necessary unless the animal is vicious.

4. The wool on the left side of the neck is clipped closely with scissors and alcohol applied.

5. The operator then grasps the neck low down with the left hand, and by means of the thumb exerts pressure over the base of the neck. The external jugular vein will be found in a groove between the omohyoid and sternomastoid muscles Firm pressure over the base of the neck usually distends the vein, which may be seen or easily felt. After locating the vein the pressure should be released for an instant, when the distention will disappear. In this way the operator may be more certain that he has located the vein.

6. A sterile stout needle, at least 2 inches in length and provided with a trocar and special shank for firm grasping, is passed quickly into the distended vein in an upward and inward direction (Fig. 38) It is essential that the needle be sharp, otherwise it will be turned aside by the wall of the vein. The end of the needle must not have too long a bevel, or the point will pierce the opposite wall before the body of the needle is well within the vein. The trocar is now removed, and blood collected in a flask or bottle and defibrinated with glass beads or rods. A short piece of rubber tubing may be attached to the needle. A suction apparatus is not needed because the flow of blood is good so long as pressure is preserved over the vein at the base of the neck.

7. When the required amount of blood has been secured, pressure is released and the needle quickly withdrawn. Bleeding ceases at once, and the neck is then washed with alcohol.

8. By this method the same vein may be used over and over again for several years. I have never known infection to occur, although the gradual formation of scar tissue about the site of puncture may interfere with the operation.

Horse and Cow.—1. Clip the hair on the side of the neck over the jugular vein.

2. Apply alcohol.

3. Apply pressure with thumb until the vein becomes prominent.

4. Insert a No. 16 to 18 gauge needle first through the skin and then into the vein.

5. After collecting blood, remove needle and wipe with alcohol.

Hog.—1. Cleanse the tip of the tail and wipe with alcohol.

2. Lay the end of the tail on a block of wood and chop off about one-quarter to one-half inch with razor.

3. Allow the blood to run into a sterile container.

4. When required amount is obtained, tie a string around the tip of the tail to prevent further bleeding.

Dog.—1. Muzzle and lay on side

2. Clip hair and then shave the side of the neck over the jugular vein

3. Have the head extended and apply pressure over jugular vein until it becomes prominent. If the tissues around the vein are loose it is well to

FIG 38—METHOD OF BLEEDING A SHEEP FROM THE EXTERNAL JUGULAR VEIN

A The needle here shown is reduced to a little more than half the actual size (From Kolmer, *Infection, Immunity and Biologic Therapy*, W B Saunders Co)

draw the tissues down at the same time the pressure is applied, thus rendering the vein immovable.

4. Insert the needle through the skin and then into the vein.

Fowl.—FROM THE COMB.—Clip off a small piece of one of the points of the comb. Sufficient bleeding will occur to furnish blood for blood counts, hemoglobin estimations, smears, etc.

FROM THE HUMERAL VEIN.—1. Hold the bird on its side so that the breast is presented toward the operator. Turn the top wing back.

2. Insert the needle (No. 21 gauge) into the humeral vein which is situated in the loose fascia on the inner side of the wing in the humeral region.

3. Withdraw the blood and place in tube as quickly as possible as the blood of fowls coagulates rapidly.

POSTMORTEM EXAMINATIONS

1. Autopsies should be performed as soon after death as possible, especially if bacteriological examinations are required.

2. Select suitable instruments and have a separate set for each step if bacteriological examinations are to be made (in which case boil all instruments for at least five minutes).

3. Fasten the animal on a board or tray exposing the ventral surface. Disinfect the hair with 1 per cent cresol and examine the skin at the point of inoculation for ulcerations, etc.

4. Incise the skin from the neck to the pubis. Cut the skin at right angles at the ends of the cut.

5. Separate the skin and lay the flaps back on each side, exposing the entire abdomen and thorax. Note the condition of the subcutaneous tissue, axillary and inguinal lymph glands

6. Make cultures of the peritoneal fluid at this time by puncturing the peritoneal wall with point of capillary pipet or needle attached to a syringe.

7. With fresh instruments cut through the peritoneal wall from diaphragm to the pubis. Make right angle cuts to form flaps which can be laid back to expose the organs.

8. Examine and make necessary cultures of the abdominal organs.

9. With blunt-pointed scissors cut through the costal cartilages, making a V-shaped incision. Lay back the flap and expose the thoracic organs.

10. If a blood culture is to be made, lift the heart and hold it in position with a hemostatic forceps. Cut the pericardium and sear an area of the right ventricle with a hot instrument; make a short incision with a sterile scalpel, withdraw blood with a sterile pipet or a sterile loop to a suitable culture medium and make smears on slides.

11. If histological examinations are desired, remove small pieces of tissue and place them in a Zenker's solution or 4 per cent formalin; use five to six times as much solution as tissue.

12. Dispose of the animal by burning or place in a 1 per cent solution of crude cresol for disinfection.

CHAPTER III

SUGGESTIONS FOR ROUTINE AND SPECIAL LABORATORY EXAMINATIONS

Principles.—1. In hospital as well as in private laboratories it is helpful and time-saving to have an understanding concerning the tests to be made when a specimen is accompanied by a request marked for "routine examination." It is hoped that the following chapter will prove helpful in deciding upon the examinations to be made as routine, and those to be conducted only upon special request, grouped as special examinations.

2. Practicing physicians do not always realize the very large amount of time and work involved in some examinations, and unless the pathologist happens to know considerable of the medical status of a patient, he is not in position to conduct the examinations of particular value. A general request for a bacteriological examination of feces, for example, can be a needlessly prolonged and laborious undertaking if only the presence or absence of typhoid bacilli happens to be the information really desired.

3. Routine examinations should be sufficiently comprehensive to give an immediate general survey. For example, a routine examination of the blood in some hospitals is confined to a hemoglobin estimation and red and white corpuscle counts but the survey is hardly sufficient without a differential leukocyte count, which also permits an inspection of the corpuscles for any abnormal types which would not be detected in the total counts. Available facilities and the question of cost usually enter into the decision.

4. In submitting laboratory reports it is helpful and time-saving to have rubber stamps or printed forms to cover the routine examinations; this lessens the risk of omitting a test and saves considerable writing.

5. As negative statements sometimes have as much value as positive ones, it is recommended that in routine examinations a report be made on the absence of substances or the negative outcome of tests. For example, if a urine contains no casts the report should read "none" instead of leaving a blank space and the physician in doubt as to whether or not the laboratory found any or overlooked reporting their presence.

6. The routine examinations to be made of hospital or private patients vary greatly in different localities and in the specialties, but it is recommended the following constitute the minimum in institutional work:

Routine blood examination
Routine urine examination

Routine blood chemistry
Wassermann and precipitation tests for syphilis in chronic disease
Stools for blood and ova (especially in warm climates)
Nose and throat cultures in children for diphtheria bacilli
Sputum (if present) for tubercle bacilli
Vaginal smears of children for gonococci
Blood grouping in certain surgical cases

7. Requests for needless laboratory work should be avoided, especially in hospital work. This is particularly true of urine and blood examinations, notably the latter when it is remembered that a goodly part of an hour is required for an accurate hemoglobin estimation, red and white count and differential. The practice of making specific requests should be encouraged, as this will leave more time for conducting fewer and more valuable examinations more thoroughly and give laboratorians the knowledge that their work is really required and that the results will be carefully noted instead of merely filed, overlooked and forgotten.

BLOOD EXAMINATIONS

Routine Examination
Hemoglobin estimation
Erythrocyte count
Leukocyte count
Differential leukocyte count (to include the nuclear index)

Special Examinations
Inspection of erythrocytes for abnormal types
Inspection of leukocytes for abnormal types
Volume of erythrocytes
Volume index
Color index
Saturation index
Peroxidase reaction
Platelet count
Reticulocyte count
Malaria and other parasites
Coagulation time
Bleeding time
Prothrombin time
Calcium time
Clot retraction time
Tonicity or resistance of erythrocytes
Sedimentation time of erythrocytes
Icterus index

URINE EXAMINATIONS

Routine Examination
  Color
  Transparency and sediments
  Reaction
  Specific gravity
  Qualitative albumin
  Qualitative sugar
  Acetone (if sugar is present) ; recommended for all urines of children
  Microscopical examination for
      Casts of various kinds
      Cylindroids
      Epithelium of various kinds
      Leukocytes
      Erythrocytes
      Spermatozoa
      Crystals of various kinds
Special Examinations
  Total solids
  Quantitative albumin
  Quantitative sugar
  Diacetic acid (if sugar and acetone are present)
  Beta-oxybutyric acid (if sugar and acetone are present)
  Quantitative chlorides
  Proteoses
  Bence-Jones protein
  Lactose
  Pentose
  Urea
  Indican
  Ammonia nitrogen
  Total nitrogen
  Bile pigments and diazo reaction
  Bile acids (qualitative and quantitative)
  Occult blood
  Hematoporphyrin
  Bacteriological examination (see Bacteriological examinations)
  Kidney functional tests

SPUTUM EXAMINATIONS

Routine Examination
  Consistency and character
  Color
  Layer formation (if kept in tall glass)
  Microscopical examination of smears for tubercle bacilli and other
      organisms

Special Examinations
  Coagulation
  Reaction

Special Examinations, *Continued*

 Specific gravity
 Microscopical examination of smears for
  Eosinophils
  Pus
  Erythrocytes
  Kinds of epithelium and "heart-failure cells"
  Actinomyces, molds, yeasts and animal parasites
  Spirochetes and fusiform bacilli
  Curschmann's spirals
  Dittrich's plugs
  Elastic tissue
  Charcot-Leyden crystals
  Myeline globules
  Cultures for pneumococci, streptococci, etc.
  Pneumococcus typing
  Guinea-pig inoculation for tubercle bacilli
  Chemical examination for albumin and urea

GASTRIC ANALYSIS

Routine Examination
 Amount of residuum or test meal (if all is removed at one time)
 Odor
 Undigested food
 Mucus
 Pus
 Bile
 Blood (including chemical tests for occult blood)
 Total acidity
 Free hydrochloric acid
 Lactic acid and rennin when hydrochloric acid is absent
 Occult blood
 Microscopical examination for
  Erythrocytes
  Leukocytes
  Mucus
  Epithelium
  Tissue fragments
  Starches
  Fats
  Crystals
  Parasites and ova
  Oppler-Boas bacilli
Special Examinations
 Protein estimation of each fraction
 Combined hydrochloric acid and acid salts
 Bile
 Enzymes
  Pepsin
  Trypsin
  Rennin

BILE AND DUODENAL CONTENTS

Routine Examination
  Amount
  Color
  Transparency
  Flakes
  Precipitates
  Mucus
  Blood
  Duodenal juice, chemically for total acidity and free hydrochloric acid
  Microscopical examination for
    Kinds of epithelium
    Leukocytes
    Mucus
    Crystals
    Bacteria in wet preparations
    Parasites

Special Examinations
  Duodenal enzymes
    Trypsin
    Amylase (amylopsin)
    Lipase (steapsin)
  Bacteriological examination by smears and culture (see Bacterio-
    logical examinations)

FECES EXAMINATIONS

Routine Examination
  Quantity
  Reaction
  Form, consistency, and gas
  Color
  Mucus
  Concretions
  Parasites
  Foods and curds
  Blood (macroscopically and chemically)
  Microscopical examination for
    Foods
      Fats (neutral fats, fatty acids, soap)
      Starches and cellulose
      Muscle (striated and not striated)
    Connective tissue
    Leukocytes
    Erythrocytes
    Epithelial cells
    Crystals
    Ova and parasites

Special Examinations

Urobilin
Bilirubin
Pancreatic ferments
Quantitative fats
Schmidt's test diet and nuclei test
Bacteriological examination (see under Bacteriological examinations
for Amebae)
Concentration methods for ova

EXUDATES AND TRANSUDATES

Routine Examination

Amount
Color
Character
Serous
Fibrinous
Purulent
Hemorrhagic
Chylous
Putrid, etc.
Coagulation
Differential cell count (cytodiagnosis)
Bacteriological examination
Smears for tubercle bacilli or other organisms
Cultures

Special Examinations

Albumin determination
Total cell count
Guinea-pig inoculation for tubercle bacilli

CEREBROSPINAL FLUID

Routine Examination

Pressure (if taken)
Amount submitted
Color
Transparency
Coagula and sediments
Total cell count
Differential cell count (omitted if fluid is clear)
Qualitative test for protein (globulin)
Qualitative test for sugar
Colloidal gold, mastic or benzoin tests
Wassermann test
Bacteriological examination by
Smear
Culture

Special Examinations
  Specific gravity
  Quantitative protein
  Quantitative sugar
  Quantitative chlorides
  Mayerhofer reduction test
  Animal inoculation and cultures for tubercle bacilli

Special Routine for Specimens Submitted for the Wassermann Test
  Pressure (if taken)
  Coagula
  Total cell count
  Qualitative protein (globulin)
  Colloidal gold, mastic or benzoin tests
  Wassermann test

BACTERIOLOGICAL EXAMINATIONS

Routine Examinations

  Pus
    Smears and aerobic cultures
    Anaerobic cultures in gangrene

  Pleural, pericardial and peritoneal fluids
    Smears and cultures for streptococci, pneumococci, etc.
    Smears for tubercle bacilli

  Sputum
    Smears for tubercle bacilli

  Nose and throat
    Smears and cultures (preferred) for diphtheria bacilli and other
      bacteria
    Smears for Vincent's angina

  Ear and mastoid
    Smears and cultures (preferred) for pneumococci, streptococci, etc.

  Nasal accessory sinuses
    Smears and cultures for pneumococci, streptococci, etc.

  Eye
    Smears and cultures (preferred) for pneumococci, streptococci, etc.
    Smears for gonococci

  Gums
    Smears for spirochetes, fusiform bacilli, *Endamoeba gingivalis*
    Cultures for streptococci, etc.

  Teeth
    Cultures for streptococci, staphylococci, etc.

  Cerebrospinal fluid
    Smears and cultures for meningococci
    Smears for tubercle bacilli

Routine Examinations, *Continued*

Blood
Cultures for typhoid fever, undulant fever, streptococci, staphylococci, meningococci, etc.

Urine
Cultures (catheterized specimens only) for *B. coli*, etc.
Smears for tubercle bacilli

Bile
Cultures for *B coli,* streptococci, etc.
Smears for bacteria, giardia, etc.

Feces
Cultures for streptococci and general aerobic flora

Special Examinations

Pus
Smears and cultures for anthrax bacilli, pathogenic fungi, etc.
Smears and animal inoculation for tubercle bacilli

Pleural, pericardial and peritoneal fluids
Guinea-pig inoculation for tubercle bacilli

Sputum
Concentration methods for tubercle bacilli
Guinea-pig inoculation for tubercle bacilli
Mouse inoculation for pneumococcus typing

Nose and Throat
Smears for leprae bacilli
Smears and guinea-pig inoculation for tubercle bacilli
Cultures for meningococci, *B. pertussis,* etc.

Cerebrospinal fluid
Guinea-pig inoculation for tubercle bacilli

Feces
Cultures for
Typhoid
Paratyphoid
Dysentery bacilli
B. abortus, etc.
Smears for *Endamoebae histolytica,*

Genital sores
Smears and fresh preparations (preferred) for *Sp. pallida*
Cultures for Ducrey's bacillus
Smears for Leishmann-Donovan bodies, etc.

Skin
Smears and cultures of furuncles for anthrax bacilli
Smears and cultures for pathogenic yeasts and fungi

SEROLOGICAL EXAMINATIONS

Routine Examinations
  Complement-fixation test for syphilis
  Precipitation test for syphilis as a control
  Agglutination tests for transfusion
    Grouping of patient
    Direct matching tests

Special Examinations
  Complement-fixation tests for
    Tuberculosis
    Gonococcus infections
    Typhoid fever
    Echinococcus disease
    Glanders, undulant fever, etc.

  Agglutination tests for
    Typhoid fever
    Paratyphoid fevers
    Undulant fever
    Tularemia
    Typhus fever, etc.

BLOOD CHEMISTRY

Routine Examination
  Urea nitrogen
  Creatinine (if urea nitrogen is increased)
  Dextrose

Special Examinations
  Uric acid
  Cholesterol
  Total calcium
  Inorganic phosphorus
  Van den Bergh or other liver functional tests
  Sugar tolerance determination (unnecessary when the fasting blood
    sugar is 0.150 milligram per 100 c.c. or higher)
  Carbon dioxide combining power of the plasma
  Chlorides

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CHAPTER IV

METHODS FOR THE PREVENTION AND EMERGENCY TREATMENT OF LABORATORY ACCIDENTS

The modern laboratory worker is surrounded by many dangers, especially from infection in the conduct of necropsies and the handling of infectious material in bacteriological examinations, as well as from breaking glassware, scalds, burns, the accidental swallowing of corrosive poisons, the inhalation of poisonous fumes, etc. This is especially true of inexperienced workers who are likely to be ignorant of potential dangers or engage in conversation and other distractions while handling and measuring infectious material, cultures, acids, alkalis, etc.

PREVENTION OF ACCIDENTS

1. Good rubber gloves always should be worn in the conduct of necropsies of animals or individuals and the handling of fresh infectious tissues (anthrax, syphilis, tuberculosis, etc.) ; and great care should be taken against accidental pricking of the fingers with sharp edges of bone and needles as well as against cuts with knives, saws, etc.

2. Pipets employed for transferring or measuring cultures of virulent bacteria like tubercle, diphtheria, and typhoid bacilli, *B. abortus,* etc., should have the mouth ends plugged with cotton or a piece of rubber tubing with a glass mouthpiece should be attached for filling and expelling. Various syringes are also available. Likewise in pipeting acids, alkalis and other poisonous solutions, the worker should use these precautions and exercise great care. It is particularly important to keep one's mind on the work and not to engage in conversation or other distractions.

3. The hands should be kept free of cuts and abrasions, particularly around the finger nails, and carefully washed with soap and water followed by immersion in a disinfecting solution after handling infectious material and before meals.

4. Table tops should be carefully wiped with a disinfectant solution after working with infectious material and it is sometimes advisable to work on a towel wrung out in a disinfectant like 2 per cent lysol or tricresol solution.

5. Pipets, test tubes, instruments, etc., employed in the examination of infectious material should be placed immediately in a disinfectant solution like 2 per cent lysol or tricresol or immediately sterilized by boiling.

6. In grinding dried bacteria a mask should be worn or the procedure conducted in a special hood insuring against the inhalation of dust.

7. Containers contaminated with sputum, feces, etc., or slides soiled with excessive amounts of vaginal or urethral pus, etc., should not be handled at all but consigned to a disinfecting process.

8. Chemical work involving the production of irritating and dangerous fumes should be always conducted under hoods with good exhausts and ventilation.

9 Electric heaters and other electric apparatus should be frequently inspected and immediately repaired as a safeguard against short circuiting and fires. Bunsen burners should never be used around inflammable material. Ether, alcohol and the like should be carefully kept away from all possible contact.

10. All laboratory workers should be immunized against diphtheria if Schick-positive; also against typhoid and paratyphoid fevers. Cowpox vaccination is advisable every few years and after every contact with smallpox. Good general health should be maintained at all costs as an effective means for keeping natural immunity and resistance at a high level.

EMERGENCY TREATMENT OF ACCIDENTS

Cuts and Needle Pricks.—1. Remove all foreign matter, such as pieces of glass, dirt, etc.; then apply 3.5 per cent tincture of iodine, taking care that the tincture reaches all crevices of the wound.

2. If the cut is slight or does not bleed copiously, bandage, placing a small pad of gauze directly over the wound and bandaging tightly enough to stop the flow of blood. If a small cut does not stop bleeding from the pressure bandage alone, apply peroxide of hydrogen and bandage with a pad of gauze over the wound.

3. If the cut is severe and bleeds copiously, apply a tourniquet to check the bleeding. If the cut is in an artery, indicated by the blood being a bright scarlet and flowing in an intermittent stream, the tourniquet is to be placed between the cut and the heart. If in a vein, shown by dark, purplish blood, the tourniquet should be placed between the capillaries and the wound. Under no conditions should a tourniquet be allowed to remain in place for more than two hours at a time.

4. Needle pricks should be squeezed to promote bleeding, carefully washed with hot water and soap and treated with tincture of iodine or 1 : 500 metaphen or mercurophen solutions.

Burns.—From Flames or Hot Objects.—1. Dress with butesin picrate ointment, and, if serious or covering a large area, bandage over the dressing. Dressing should be completely changed at least once a day.

2. Blisters forming from burns should be opened and drained by puncturing in at least two places near the edge and pressing out the liquid. The puncture may be made with a flame-sterilized needle or razor blade. When changing dressing, any blisters present should be again drained.

FROM CHEMICAL AGENTS.—1. Burns from *strong acids,* bromine, chlorine, phosphorus or other material of acid character, are washed first with large quantities of water, then with 5 per cent sodium bicarbonate or 5 per cent ammonium hydroxide solution, dressed and bandaged as above.

2. Burns from *strong alkalis,* sodium hydroxide, metallic sodium or potassium or other materials of alkaline nature, are washed first with large quantities of water, then with 5 per cent boric or acetic acid solution, dressed or bandaged as above.

3. Burns from *phenol* are washed immediately with strong alcohol, then dressed and bandaged, if necessary.

In the Eye —Flush first with large amounts of water. Then, if due to *acid* material or formaldehyde, flush with 5 per cent sodium bicarbonate solution. If due to *alkaline* material, flush with 5 per cent boric acid solution. In either case a drop or two of castor, cotton seed, or olive oil is allowed to remain in the eye as a soothing agent.

Scalds.—Blisters that have formed are to be drained as above, then the injury is dressed and bandaged, using suitable neutralizing agents if due to acid or alkaline materials, as prescribed for burns.

Swallowing of Mineral Acids.—1. Wash out the mouth immediately with large amounts of water and alkaline solutions like decinormal sodium hydroxide, milk of magnesia, etc.

2. Give calcined magnesia, white magnesia, milk of magnesia, or lime-water *immediately,* mixed in milk or any mucilaginous fluid that will act as a demulcent. Repeat the dose at short intervals until it may be inferred the poison is neutralized. Do not give carbonates as antidotes for mineral acids. Oleaginous and mucilaginous fluids should be given freely, even as vehicles for the antidotes. In the case of strong sulphuric acid, water, if given at all, should be given in large quantities, on account of the heat developed. Ice may be given to relieve thirst and pain. Stomach tube and emetics should not be used. Or give a teaspoonful of *universal antidote* in a small glass of warm water. This antidote is made by mixing two parts of pulverized charcoal, one part of magnesium oxide and one part of tannic acid. It is well to keep a supply on hand.

Swallowing of Caustic Alkalis.—1. Wash out the mouth immediately with large amounts of water and weak acid solutions.

2. Administer a weak acid, such as 5 per cent acetic acid, vinegar, or lemon juice, until it may be inferred the alkali has been neutralized; or give a teaspoonful of universal antidote (see above) in a small glass of warm water. Give butter, olive or cotton seed oil, or other oils or fats to form soaps.

3. Assist vomiting by drafts of tepid water.

Swallowing of Phenol and Phenolic Compounds.—1. Wash out the mouth immediately with dilute alcohol (30 to 40 per cent).

2. Give *immediately* 4 ounces of alcohol mixed with 4 ounces of water, or one half pint of whisky, and remove from stomach by use of emetic, prefer-

ably of mustard (tablespoonful in enough water to make a thin cream). If stomach tube is used, it must be with great care.

Inhalation of Corrosive Gases.—1. Remove patient to fresh air, and place prone, face down, with head slightly lower than the chest, so that vapors may drain from the lungs.

2. Permit inhalation of vapors of acetic acid if ammonia is active agent, and of dilute ammonia if acid vapors are being treated.

3. Inhalation of vapors of alcohol or ether soothes the respiratory tract.

4. *Toxic headaches* due to the inhalation of vapors of various materials may be combated by removal to fresh air, administration of 5 to 10 grains of aspirin, and allowing the patient to rest for a time.

5. For *hydrogen sulphide* fumes inhale ammonia from 5 per cent ammonium hydroxide, or inhale fresh air containing a small proportion of chlorine. Administer milk, white of egg in water, olive or cotton seed oil, etc.

Swallowing of Virulent Cultures.—1. Accidental contaminations of the mouth with cultures of staphylococci, streptococci, pneumococci, etc., are not dangerous although it is advisable to immediately rinse well with hot water and disinfectant like 1 : 5000 metaphen or mercurophen, 1 : 2000 bichloride of mercury, diluted hydrogen peroxide, etc.

2. Virulent diphtheria cultures are much more dangerous. The above measures should be immediately employed. If Schick-negative, there is little or no danger of infection. If Schick-positive, infection may follow unless a prophylactic injection of 1000 units of antitoxin is taken.

3. The swallowing of typhoid, cholera and dysentery cultures also carries some danger, especially in the case of freshly isolated cultures. The mouth should be immediately disinfected as described above. In the case of typhoid cultures, it may be advisable to undergo vaccination unless this had been done within two years.

4. Great care is also required in working with *B. abortus* (*Micrococcus melitensis*). In case of contamination immediate immunization with vaccine is recommended following the cleansing measures recommended above.

Contamination with Syphilitic Material.—1. In case of needle pricks or cuts while removing chancre or other syphilitic material for dark-field or other examinations, squeeze to promote bleeding; then wash carefully with *hot* water and *plenty of soap*. Dry and thoroughly apply 33 per cent ointment of calomel or metallic mercury or 1 : 1000 solution of bichloride of mercury (ointments preferred). Renew the applications twice a day for at least three days.

2. Contamination of the hands with the blood of syphilitic patients while taking blood for the Wassermann test, giving injections, etc., is rarely dangerous and not at all unless there are needle pricks, cuts or abrasions. We have never seen or heard of accidental infection in this way. In case of contamination of cuts and pricks with blood, it is advisable, however, to use the measures described above.

3. We have never heard of or seen syphilitic infection following accidental

contamination of the mouth with syphilitic serum or cerebrospinal fluid while conducting the Wassermann and other tests. The manipulations involved in the preparation of serum and especially heating at 55° C. for ten minutes or longer, are almost sure to kill the spirochetes, even if they were present, as *Spirochaeta pallida* is very susceptible to heat.

Spinal fluids, however, especially from paretics, are potentially more dangerous, particularly if contamination occurs during spinal puncture or soon thereafter. If needle pricks or cuts occur, the measures given above should be applied. If spinal fluid is accidentally taken into the mouth during total cell counts or other examinations, it will suffice to rinse thoroughly with water and 1 : 5000 metaphen or mercurophen or 1 : 1000 bichloride of mercury.

SECTION II

CLINICAL PATHOLOGICAL METHODS

CHAPTER V

METHODS FOR THE EXAMINATION OF THE BLOOD

APPARATUS FOR BLOOD EXAMINATIONS

Principles.—Next to urine examinations it is probable that red and white corpuscle counts with hemoglobin estimations and differential leukocyte counts are the most frequently conducted laboratory examinations in clinical pathology. Yet a comparison of the counts on the same patient at approximately the same time by different workers is apt to show widely different results due to inaccuracies in technic and apparatus. Cheap diluting pipets, counting
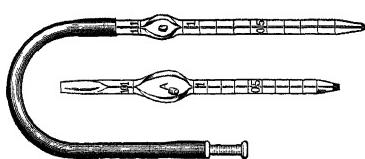
FIG. 39.—THOMA DILUTING PIPETS.	FIG. 40.—TRENNER AUTOMATIC PIPETS
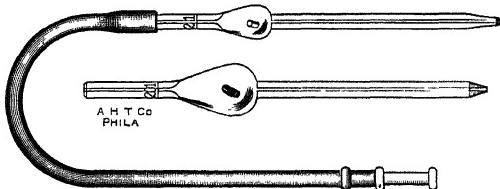
chambers and cover glasses are responsible for a great deal of error and only the better grades are recommended, preferably with the United States Bureau of Standards certification. Pipets with broken tips are especially likely to be inaccurate and it is recommended that only the best apparatus be employed with scrupulous attention to technical details.

1. Thoma (Fig. 39) or Trenner (Fig. 40) diluting pipets are recommended for red and white corpuscle counting, the latter particularly for inexperienced workers if difficulty is experienced in stopping the flow of blood exactly at the 0.5 mark on the capillary stem.

2. All pipets should be guaranteed to be within the tolerance for accuracy established by the United States Bureau of Standards of ± 3 per cent for the principal interval. Pipets with the Bureau of Standards certification are recommended, although probably too expensive for routine work. It is advisable, however, for every laboratory to have at least one certified red corpuscle

and leukocyte pipet with which new pipets may be compared before the latter are accepted for work.

3. While many different counting chambers are in use, the Levy with the improved Neubauer ruling (Fig. 41) is recommended; also the Levy-Hausser with bakelite holder and double Neubauer ruling. At slight extra cost these may be purchased with the United States Bureau of Standards certification and every laboratory should have at least one for comparative counts with new chambers before the latter are adopted into routine work.

4. Accurate blood cell counting requires a cover glass with optically plane surfaces. *The curvature of an*
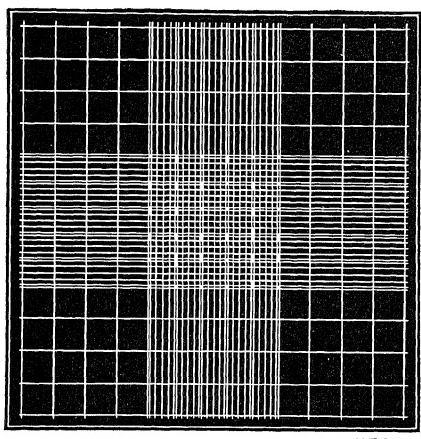
FIG. 41.—ENTIRE AREA OF IMPROVED
NEUBAUER RULING.
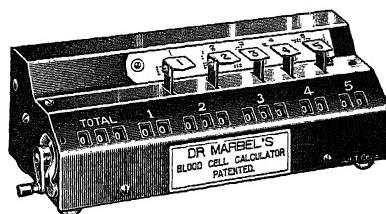
FIG. 42.—MARBEL BLOOD CELL
CALCULATOR.

ordinary cover glass as used in bacteriological work is such as to render an otherwise perfect counting chamber absolutely useless from the standpoint of accuracy. For very little extra cost, cover glasses may be obtained with the United States Bureau of Standards certification, and these are recommended for the best work. The Hausser reënforced precision glasses are recommended for the counting chamber method of standardizing vaccines.

5. The Marbel blood cell calculator (Fig. 42) is highly recommended for red and white corpuscle counting and for differential leukocyte counting.

FIG. 43.—SIMPLE BLOOD
LANCET.

METHODS FOR OBTAINING BLOOD

1. Blood is usually obtained by pricking a finger or the lobe of an ear. The latter is supposed to be more vascular with a smaller prick required but the finger is to be preferred.

FIG. 44.—SPRING BLOOD
LANCET.

2. All apparatus (pipets, diluting solutions, slides and hemoglobinometer) should be in readiness.

3. The simple (Fig. 43) and the spring (Fig. 44) lancets are quite service-able although a straight Hagedorn needle No. 6 ground to a short angle is quite satisfactory. The "sticker" should be broad rather than narrow, so that an ordinary needle or pin is unsatisfactory.

4. The finger should be warm with a free circulation of blood (immersion in warm water may be required). If cold and clammy, too much squeezing is required, with resulting error.

FIG 45—PRICKING SIDE OF FINGER WITH HAGEDORN NEEDLE
(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

5. Wash off the side of the last phalanx or the end of the middle finger with alcohol and *thoroughly dry   Do not prick a wet finger.*
6. Squeeze to steady the part and lessen the pain sense while making a quick prick (Fig. 45).
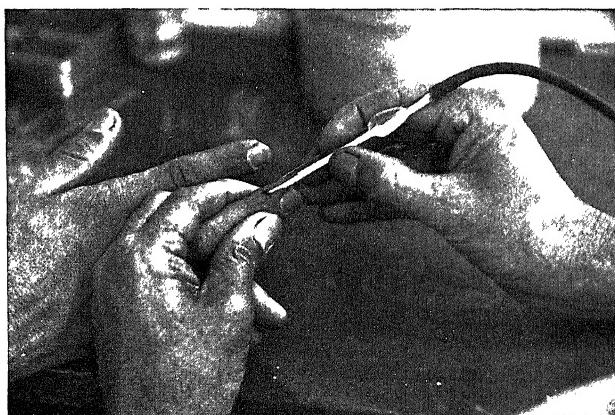
FIG 46—TAKING UP BLOOD INTO PIPET FROM PATIENT'S FINGER
Note that the operator's right hand is steadied by touching a finger against the finger of the patient   (From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

7. If the tip of the finger is used, lance across rather than parallel with the lines of the skin.

8. Do not squeeze too hard near the prick as this will dilute the blood with
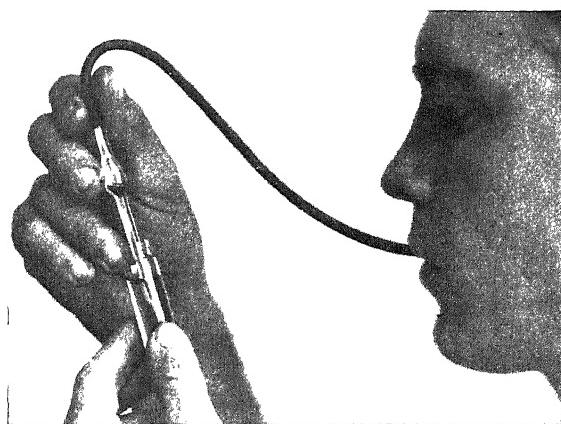
FIG 47—FILLING PIPET WITH DILUTING FLUID

The pipet is held high enough so that the marks are level with and are plainly visible to the eye   (From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

tissue "juice" and lead to error.   The prick should be deep enough to give a free flow with gentle squeezing one-fourth to one-half inch from the site.

9. Wipe away the first drop or two of blood.

10. It is advisable to first take blood for the hemoglobin estimation fol-
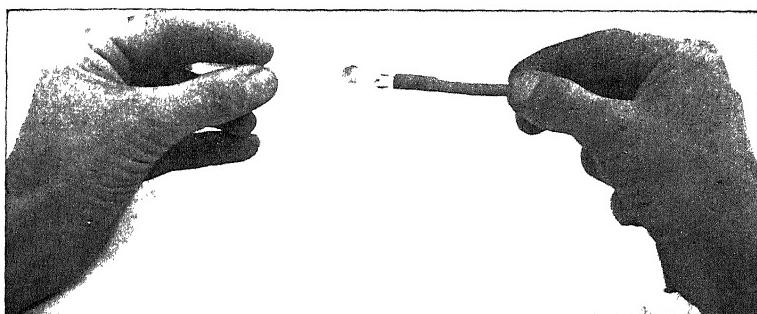
FIG 48—REVOLVING PIPET TO MIX CONTENTS

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

lowed by the red and white corpuscle pipets and the preparation of smears for differential leukocyte counts.

11. Well up a good-sized drop of blood for each pipet and especially for the leukocyte pipet, which requires much more than the red corpuscle pipet (Fig. 46).

12. Be sure to fill each pipet accurately with the proper diluting fluid immediately after taking blood (Fig. 47).

13. Immediately after filling a pipet with diluent shake it for thorough mixing (Fig. 48).

14. If the pipets are to be carried any considerable distance to the laboratory, stretch a rubber band over the ends (Fig. 49) or one of the special devices (Figs. 50 and 51) to prevent the contents from escaping.
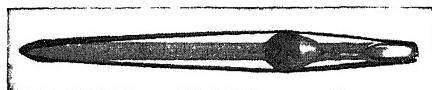
FIG 49—PIPET SEALED WITH A BROAD
RUBBER BAND

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

FIG. 50—THE TRENNER CLOSURE.

15. Prepare two or more smears on *perfectly clean grease-free and polished slides* [1] Touch the end of a slide to a large drop of blood as shown in Figure 52; then spread the drop with a second slide as shown in Figures 53 and 54. As soon as the blood has spread entirely across the end of the spreader slide (held at an angle of about 45 degrees) with a rather quick movement, *push* (do not pull) the spreader toward the other end of the under slide. If made too slowly the spread will be too thin. Allow the blood to dry.

16. Blood smears may also be made on cover glasses. They should be perfectly clean and free of grease. Take up a small drop of blood on one without touching the surface of the skin and place it on the second in such manner that the corners do not overlap. As soon as the blood spreads out
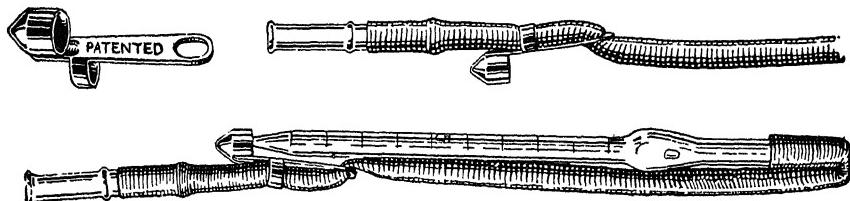
FIG 51—THE DRUMMOND PIPET HOLDER

between the glasses draw them apart in a plane parallel to their surface. Dry in the air. This method is preferred by many clinical pathologists although it tends to produce rupture of the leukocytes.

17. A combination of the slide and cover-glass methods is that of Beacom in which smears are prepared as follows: A small drop of blood is placed

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[1] Place slides in glacial acetic acid for fifteen minutes; wash in distilled water; place in 95 per cent alcohol for five minutes; dry with clean towel; pass through Bunsen flame before using
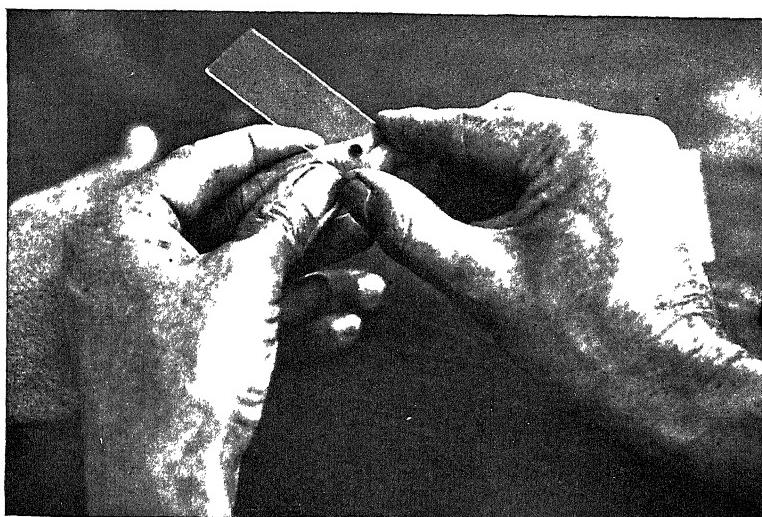
FIG. 52.—TAKING BLOOD FOR A SMEAR.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore)
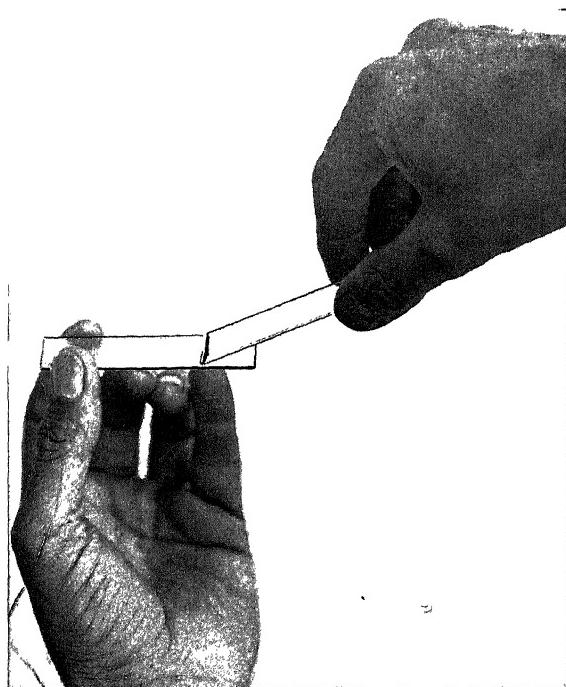
FIG 53.—SLIDES HELD IN PROPER POSITION FOR SPREADING BLOOD

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore)

near the end of a clean slide and a cover glass is dropped on; if the blood does not flow out properly, very gentle pressure may be used. The slide is then held on a table with the left hand, and the first two fingers of the right are placed near the edges close to the left end of the cover glass. Then with an even, fairly rapid pull it is slid along the slide in the direction of its long
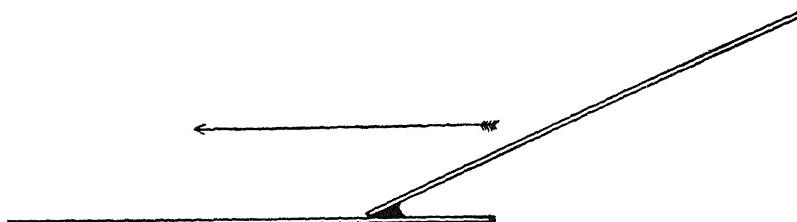
FIG. 54.—PROPER ANGLE FOR HOLDING SLIDES.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore.)

axis with only enough pressure to keep the fingers from slipping from the spreader. This leaves a smooth, even smear on the slide for examination.

METHOD OF USING OXALATED BLOOD FOR HEMATOLOGICAL EXAMINATIONS

1. According to Osgood, Haskins and others, oxalated blood may be used for the following examinations if the latter are made within the designated hours after collection:

	Hours
Hemoglobin estimation	24
Red cell count	24
Platelet count	3
Red cell volume	23
Color index	24
Volume index	3
Saturation index	3
Icterus index	4
Van den Bergh test	4
White cell count	24
Making the smear for differential count	1
Peroxidase test	3
Fragility test	3
Sedimentation rate	3

2. Prepare a month's supply of test tubes containing 2 milligrams of dry potassium oxalate per c.c. of blood to be taken, by measuring into each, with a buret, 0.1 c.c. of 2 per cent potassium oxalate per c.c. of blood to be taken and evaporating to dryness in a dry sterilizer. Keep corked. (For small labo-

ratories 1 drop of saturated potassium oxalate is sufficient for 10 c.c. of blood and has a negligible dilution effect.)

3. Draw blood from vein by usual technic, but tourniquet should be released if more than two minutes are required for securing sufficient blood.

4. *Remove needle* from syringe before running blood into oxalate as hemolysis will result if blood is forced through the needle.

5. Stopper test tube (never use cotton) and shake at once by holding test tube horizontally in left hand and tapping other end with right hand.

6. The blood must be thoroughly mixed in this manner *immediately* before samples are withdrawn for any test.

7. Samples should be taken directly from the test tube—not from blood poured out on a slide or watch glass.

8. The tube must be kept stoppered at all times when not in use.

9. The time limits given above should be observed if the most accurate results are desired, although as a rule a slightly longer time will not introduce clinical error.

10. If during the study of the blood further hematological work is deemed desirable, it may be done on the same sample. Repeated estimations are possible for checking results.

11. If an unusual or interesting blood picture is encountered, as many slides as are desirable can be made for future reference or for teaching purposes without again disturbing the patient.

STANDARD HEMATOLOGICAL NORMALS AND VARIATIONS

1. For the purpose of standardizing the various blood tests so that results obtained in different laboratories will be comparable to a definite standard, it has been necessary to select certain figures as *standard normals.* These do not represent the average normals but are figures usually within the normal range which can be most conveniently used for comparison and for estimating the various indices.

2. By the use of these standard normals a finding of 100 per cent hemoglobin or a volume per cent of 88 will represent the same number of grams of hemoglobin or the same number of c.c. of packed cells no matter where the tests were conducted.

3. *The standard normals given below represent 100 per cent,* which is a figure used for calculating and reporting results.

4. For the purpose of determining whether the results obtained by the various tests are normal or abnormal, a normal range is given on the following page which represents the low and high results considered within normal.

5. The standard normals are fixed and represent the unit of measure. The ranges, however, may be changed to suit the pathologist or clinician.

Examinations	Standard Normal	Normal Range
Hemoglobin (male)	17.3 gm. (100 per cent) per 100 c.c. of blood	14 to 18 gm. (80 to 105 per cent) per 100 c.c. of blood
Hemoglobin (female)	17.3 gm. (100 per cent) per 100 c.c. of blood	12 to 15.5 gm. (70 to 90 per cent) per 100 c.c. of blood
Erythrocytes (male)	5,000,000 (100 per cent)	4,500,000 to 6,000,000
Erythrocytes (female)	5,000,000 (100 per cent)	4,000,000 to 5,500,000
Leukocytes	According to age	4,000 to 16,000 (up to 17,000 in infants)
Cell volume (male)	50 c.c. (100 per cent) per 100 c.c. of blood	40 to 50 c.c. per 100 c.c. of blood
Cell volume (female)	50 c.c. (100 per cent) per 100 c.c. of blood	35 to 45 c.c. per 100 c.c. of blood
Volume per cent (male) . . .	100 per cent	80 to 100 per cent
Volume per cent (female)	100 per cent	70 to 90 per cent

EXAMINATION OF THE BLOOD OF LABORATORY ANIMALS

As various blood examinations are sometimes required in the course of medical research employing the usual laboratory animals and since veterinarians are employing blood counts with increasing frequency in the diagnosis of disease of the lower animals, a table, after Kleineberger and Carl, of average counts of normal animals commonly employed in laboratories is given on page 65 for assistance in evaluating results.

METHODS FOR CLEANING BLOOD APPARATUS

1. Pipets and counting chambers should be cleaned immediately or soon after using.

2. Draw water through pipets until all traces of blood and serum are removed.

3. Without drying, draw through alcohol or acetone.

4. Draw through ether (may be omitted if acetone is used).

5. Draw air through until the pipet is dry (if properly dried the bead in the bulb should be freely movable).

6. If blood has dried in the stem of a pipet, remove it with horsehair or fine stiff wire and fill pipet with antiformin, nitric acid or bichromate-sulphuric acid cleaning fluid and allow to stand overnight; then clean thoroughly as described.

THE BLOOD OF LABORATORY ANIMALS *

Name	Hemoglobin, Per Cent (Sahli Method)	Erythrocytes		Leukocytes, Per C.Mm.	Polymorphonuclears, Per Cent	Large Lymphocytes, Per Cent	Small Lymphocytes, Per Cent	Eosinophils, Per Cent	Basophils, Per Cent	Mononuclears, Per Cent	Transitional Leukocytes, Per Cent
		Per C.Mm.	Size (Micra)								
Mouse	92	9,725,000	5.7	7,400	46	1 5	37	1.25	....	....	0 75
Rat	105	9,300,000	6 2	15,200	16	24.5	53 5	3 5	....	0 5	2
Guinea-pig	75	5,270,000	5 4	15,000	38 5	13 5	33	13	0.85	0 5	0 65
Rabbit	50 5	5,250,000	6 3	8,150	50 5	10 5	35	1	2.5	0 15	0.35
Cat	56	7,393,000	5.7	15,600	68.5	7	18	5	....	0 04	1 46
Dog	94	7,225,000	6 8	10,000	77.86	7	8.6	4 2	0 04	0 03	2
Monkey	67	6,352,000	7.1	7,470	35 8	12	46	3.4	0 3	1 3	1 2
Sheep	59	11,800,000	4 3	6,500	34	9	49 33	6.33	0 84	0 16	0 34
Chicken	62	3,117,000	7 3×12	35,000 to 60,000 †	29.5	12 3	51 5	4 5	2.2	....	....
Pigeon	93	4,055,500	6.4×12	10,430 to 31,420	41	13 5	42	1 5	2	....	....
Frog	52	3,820,000	15.7×22 8	2,410 to 20,640	26 5	16	28 5	6	23	....	....

* After Kleineberger and Carl.
† The leukocyte count on normal chickens is subject to considerable variation. Other authors give much lower figures.

7. The multiple aspirating nozzle for attachment to a pump (Fig. 55) or the Haden cleansing apparatus (Fig. 56) are very convenient and time-saving for the simultaneous cleaning of one to four pipets.

8. The ruled area, the surface of the slide and the cover glass of the counting chamber should be cleaned immediately after use with water and dried with a soft lint-free cloth. If this is not done the lines will become partly obliterated with débris. If diluted blood has been allowed to remain on the slide or the ruling becomes indistinct, it may be necessary to immerse the slide in decinormal sodium hydroxide or in one of the solutions mentioned above for cleaning

Fig. 55.—Multiple Aspirating Nozzle.
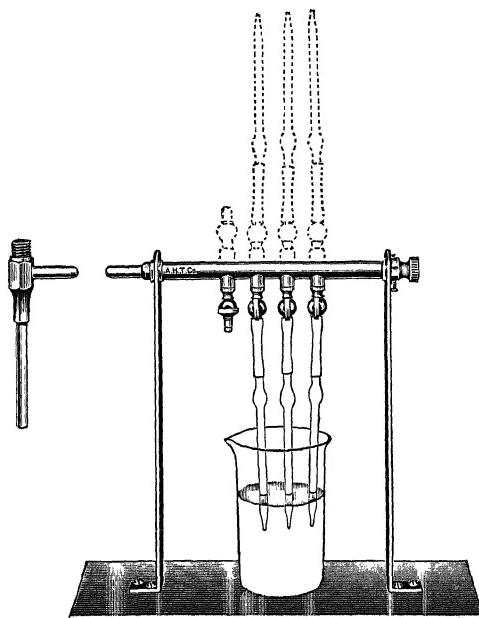
Fig. 56.—Haden Cleansing Apparatus.

pipets. The Levy-Hausser chamber can also be washed with alcohol and ether without damage.

ESTIMATION OF HEMOGLOBIN

Principles.—1. None of the methods in common use can be recommended from the standpoint of absolute accuracy. Owing to a variety of methods and standards the estimation of hemoglobin in per cent is probably of the least value in the routine blood examination. Widely varying results may be reported therefore on the same individual examined in different laboratories. *But much of this inaccuracy and misunderstanding could be overcome by expressing the results as grams of hemoglobin per 100 c.c. of blood.*

2. It is not possible as yet to recommend any simple method for routine work as a standard.

3. The most accurate method available at present appears to be that of Van Slyke, in which the oxygen capacity of the blood is determined and the hemoglobin calculated from the amount of oxygen secured and measured as the free gas in the special apparatus used for estimating the carbon dioxide combining power of plasma. This method is not adapted for routine work but should be used for checking up on the accuracy of clinical methods. Wong's method for determining the iron and hemoglobin content is sufficiently accurate for practical work and requires less time.

4. The old Tallqvist method is serviceable for a rough estimation and is described below only for this purpose.

5. Methods depending upon the conversion of hemoglobin into acid hematin (Sahli and Haden) would appear to be acceptable for routine examinations. The Dare is fairly good, providing the color disk is sufficiently accurate.

6. *It is recommended that the reports show hemoglobin in grams per 100 c.c. of blood instead of in per cent.*

7. A diminution of hemoglobin below the normal is designated as *hypocytochromia*; an increase as *hypercytochromia*.

Sahli's Method.—1. Fill the graduated tube of Sahli's hemometer with decinormal solution of hydrochloric acid to the mark 10 (Fig. 57).

2. After cleansing finger tip or lobe of ear with alcohol, make a puncture with lancet and gently compress (*the blood should flow freely*); wipe off first drop, then fill pipet to the mark 20 with blood.

3. Immediately place the blood in the graduated tube containing the hydrochloric acid solution. Remove the last trace of blood by drawing the solution up in the pipet and expelling several times.

4. Dilute the mixture in the graduated tube with water until it has the same tint and intensity of color as the standard tube. This is best done by adding a few drops at a time and comparing after each addition.

FIG. 57.—SAHLI HEMOMETER.

5. When both tubes are of the same color, note the figures on the tube at the *bottom of the meniscus*.

6. Some of the newer tubes show both per cent and grams of hemoglobin per 100 c.c. of blood.

7. The normal for adults is 13.7 to 17.3 grams per 100 c.c. of blood or approximately as follows:

Males (18 years and over).... 15.76 gm. per 100 c.c. (range 13.5 to 19)
Females (18 years and over).. 13.76 gm. per 100 c.c. (range 10.98 to 16.49)
Children (1 to 15 years)...... 10.6 gm. per 100 cc. (range 10.2 to 11.0)
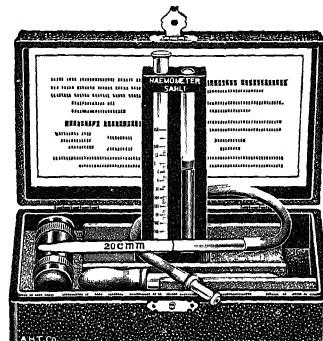
8. Accurate results demand that the inside diameters of the graduated tube and the tube containing the standard solution shall be the same and that the pipet and graduated tube be accurately marked. These specifications can be met without unreasonable extra expense. Of even more importance is the correct color of the standard solution. The tinted tubes now available are usually accurate to within 5 to 10 per cent and may be used either by day or night.

9. A new standard color tube should be checked by the Van Slyke method before being used. If there is more than 10 per cent error it should be discarded. Otherwise the error can be adjusted by adding or subtracting from the readings obtained so that 100 per cent will equal 17.3 grams of hemoglobin per 100 c.c. of blood.

Haden's Method.—This employs the new Haden-Hausser hemoglobinom-
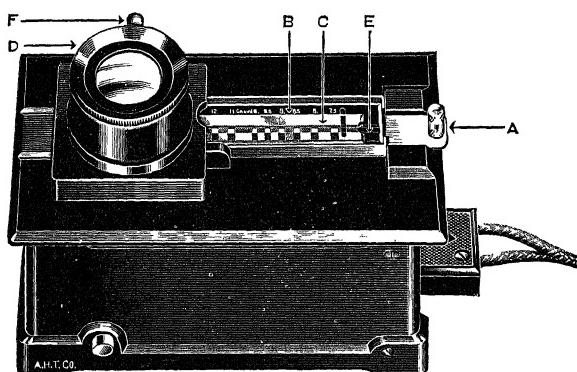
FIG. 58A.—HADEN-HAUSSER HEMOGLOBINOMETER.

A, Movable carrier; *B*, Comparator slide; *C*, Cover glass; *D*, Reading microscope; *E*, Wedge-shaped channel; *F*, Shutter.

eter (Fig. 58A and 58B) in which hemoglobin is also converted into acid hematin and the color compared with a permanent color scale of tinted glass.

1. Draw blood to the mark 0.5 of a leukocyte pipet and decinormal hydrochloric acid to 11 (gives a 1·20 dilution). If the hemoglobin is below 50, draw blood to 1 and the acid to 11 (gives a 1:10 dilution).

2. Shake well and allow to stand for a few minutes.

3. Blow out several drops and allow the blood to run into the channel of

FIG. 58B.—SHOWING DETAILS OF COMPARATOR SLIDE *B* IN FIGURE 58A.

the slide at the end of the cover glass. The channel fills by capillarity. A thin uniform film will also extend by capillarity from the dilution channel and cover the comparator slide above the color standard. This insures a light transmitting surface common to both dilution channel and standard.

4. A color-matching reading is now made according to directions accompanying the instrument and the results expressed in grams of hemoglobin per 100 c.c. of blood.

Dare's Method.—1. Electrically illuminated instruments (Figs. 59 and 60) are to be preferred to the original model using a candle.

2. Each instrument should be checked by the Van Slyke method before being used in routine work.

3. See that the porcelain and glass slides are perfectly clean and tightly clamped in holder.
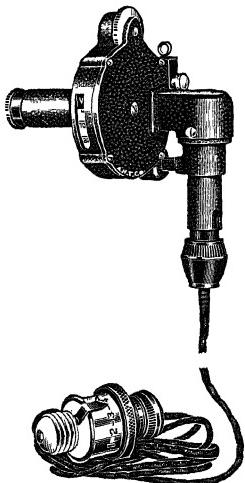
FIG. 59 —DARE HEMOGLO-
BINOMETER, OPERATING
ON 110-VOLT CIRCUIT.
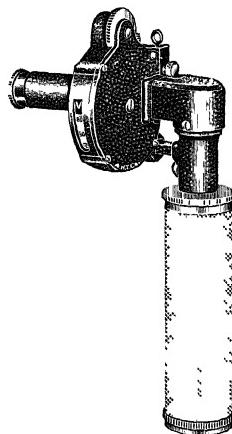
FIG 60 —DARE HEMOGLO-
BINOMETER, OPERATING
ON A BATTERY.

4. Screw the camera tube into place and see that electric bulb is in working order.

5. Fill capillary space between glass plates by placing one corner in contact with a large drop of blood.
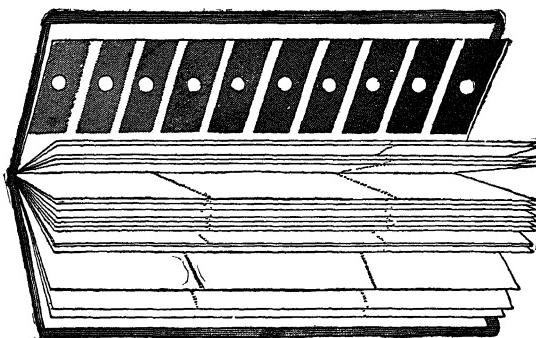
FIG. 61.—TALLQVIST HEMOGLOBIN SCALE.

6. Wipe off excess of blood and place holder in proper position in instrument.

7. If candle is used, the blue flame should be below the color aperture and the wick not over half an inch in length.

8. Examine in a dark corner of room, moving color scale until it exactly corresponds to color of blood between the glass plates.

9. Read off the number indicated at the beveled edge of the rectangular opening of the case. This figure represents the percentage of hemoglobin; 100 per cent is equal to 13.77 grams of hemoglobin per 100 c.c. of blood.

Tallqvist's Method.—1. This method is given as a rapid means for *roughly* estimating the hemoglobin but is not recommended for any other purpose on account of its wide range of error (Fig. 61).

2. Prick the finger or ear and secure large drop of blood by very gentle pressure.

3. Remove a piece of blotting paper from the Tallqvist book and place it on the drop until it is absorbed.

4. As soon as the gloss has disappeared, fold the paper back of the drop and compare the color with the printed color scale at back of the book by allowing the blood-stained portion of the paper to appear at the various holes in the scale.

5. Read the percentage at the side of the color which it best matches.

6. The readings are usually too low as compared with more accurate methods.

COUNTING ERYTHROCYTES

Principles.—1. Blood is diluted exactly 1:200 with a special pipet, using an isotonic diluting fluid for the preservation of the corpuscles. The diluted blood is then placed in a special counting chamber and the cells in 0.00025 c.mm. are counted. This figure is then multiplied by 4000 and then by 200 (the dilution factor) to obtain the number of erythrocytes in 1 c.mm. of undiluted blood. This constitutes the method for reporting the results.

2. Leukocytes are likewise present and may be distinguished from erythrocytes, but as a general rule they are included in the count; the number present, however, is ordinarily so small that the inaccuracy is of little or no importance.

3. The *normal* number of erythrocytes per c.mm. of blood is ordinarily as follows:

Men .... 4,500,000 to 6,000,000
Women .... 4,000,000 to 5,500,000

4. A pathological decrease is called *oligocythemia* or *anemia;* an increase is called *polycythemia.*

Procedure.—1. Draw blood up exactly to the 0.5 mark of the Thoma pipet marked 101. If the Trenner automatic pipet is used, draw blood by suction until the stem is nearly full and then discontinue suction and allow the blood to automatically reach the extremity.

2. Immediately draw up diluting fluid to the mark 101, thus making a dilution of 1:200 in either pipet, while rotating the pipet between the thumb

and forefinger. Use 0.85 per cent saline solution or either of the following (Hayem's preferred) :

HAYEM'S DILUTING FLUID

Water (distilled) .... 200.0 c.c.
Sodium chloride .... 1.0 gm.
Sodium sulphate .... 5.0 gm.
Mercuric chloride .... 0 5 gm.

TOISSON'S DILUTING FLUID

Neutral glycerin .... 30.0 c.c.
Water (distilled) .... 160.0 c.c.
Sodium sulphate .... 8.0 gm.
Sodium chloride .... 1.0 gm.
Methyl violet .... 0.025 gm.

3. The diluting fluid should be crystal clear and filtered if necessary to be free of artefacts.

4. The ruled area of the Levy-Hausser counting chamber and the cover glass must be carefully cleaned and absolutely free from dust or lint.

5. Place the cover glass in position over the ruled area, using gentle pressure to insure accurate adjustment. The Levy-Hausser chamber is provided with a pair of clips to prevent any movement during the count. While continuing pressure on the cover glass, slide the centrally placed clip into position simultaneously.

6. Close the tip by means of the thumb. Sharply kink the rubber tubing over the other end and place the second finger over the kinked tubing. Trenner pipets are more fragile than the Thoma pipets and when filling, cleaning or attaching rubber tubing, the capillary stem should be held between the thumb and forefinger to avoid strain on the bulb. Rotate the pipet well for several minutes, holding in a horizontal position, and finally shake sidewise.

7. Blow out six to eight drops from the pipet and *without loss of time* touch a drop to the end of the polished surface bearing the ruling, allowing the drop to flow under the cover glass. The suspension should not flow into the moats on either side, nor should any bubbles form under the cover glass.

8. Allow about three minutes for the corpuscles to settle.

9. Examine with 4 or 16 millimeter objective. Center the light and reduce its volume by lowering the condenser and partially closing the diaphragm.

10. Locate the finding line which leads to the ruled-off area. *Carefully avoid touching the cover glass with the lens, as this would disturb the corpuscles and lead to error in the count.*

11. When correctly focused, the corpuscles are sharply defined and the rulings appear as well-defined *black* lines (Fig. 62).

When incorrectly focused, the ruled furrows appear as *white* lines, and the

corpuscles, which lie above the plane of the ruled surface, are out of focus (Fig. 63).

12. The counting slide will be found to have a number of small squares marked upon it. The size of these small squares is 0.05 millimeter by 0.05 millimeter or 0.0025 sq. mm. When the cover slip is in place there is a chamber formed measuring 0.1 millimeter in depth. Therefore the small squares are in reality cubes measuring 0.00025 c.mm. (0 05 × 0.05 × 0.1 millimeter = 0.00025 c.mm.).

It is impracticable to count the number of cells in 4000 of these small cubes, which would give the number of cells per c.mm. of diluted blood, so that a smaller number of cubes should be chosen.

13. Count the number in 80 of the small squares. It will be noted that the small squares are separated into groups of 16. Five of these groups therefore
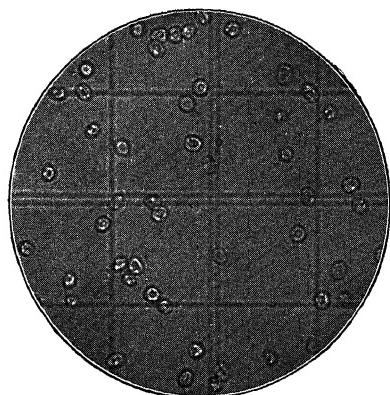
FIG 62 —CORRECT FOCUSING.   FIG. 63 —INCORRECT FOCUSING
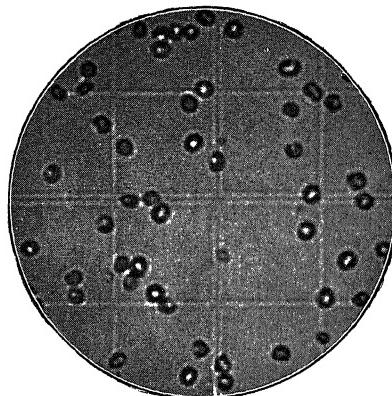
contain 80 small squares. Do not count those cells touching the lower and right-hand lines but include all touching the upper and left-hand lines.

14. Divide the number of cells found in 80 small squares by 80, thus determining the average number per small square or in 0.00025 c.mm.

15. Multiply the average number of cells per 0.00025 c.mm. by 4000 to determine the number of cells in 1 c.mm. of diluted blood, and by 200 to determine the number in 1 c.mm. of undiluted blood.

16. *Therefore in routine work count the number of cells in 80 small squares and add four ciphers.*

17. The following sources of error must be kept in mind and carefully avoided: (*a*) Inaccurate dilution due to faulty pipets or technic; (*b*) too slow manipulation, allowing a little of the blood to coagulate; (*c*) inaccuracy in the counting chamber and especially in its depth due to inaccurate cover glass, faulty manufacture, loosening of parts, etc.; (*d*) presence of yeasts and other artefacts in the diluting fluid.

ESTIMATING THE VOLUME OF ERYTHROCYTES

Principles.—1. The estimation of the volume of packed cells to the whole blood is chiefly used for the purpose of determining the volume index. It can be used as a substitute for the erythrocyte count for determining the degree of anemia. In this respect it gives results more representative of the condition of the blood than the count, due to the fact that changes in the size of erythrocytes, as well as in their number, will be detected.

2. The normal amount of packed cells to 100 c.c. of blood is as follows:

> Men................................ 40 to 50 c c
> Women............................ 37 to 45 c c

3. By the Haden-Sanford method described below, from 46 to 48 per cent are corpuscles and 54 to 52 per cent plasma.

Finger Method.—1. Prick the finger as for blood count.

2. Gently compress until a large drop of blood collects.

3. Draw the blood up in a Van Allen hematocrit tube to the mark 10 (Fig. 64).

4. Draw the diluting fluid (1.6 per cent sodium oxalate) into the bulb of the pipet until it is about one-third full. Then mix by twirling between the fingers.

5. Place the tube in holder (Fig. 65) or wide rubber bands can be stretched over the ends.

6. Centrifuge the tubes at high speed for about one-half hour. Remove the tubes and read the volume of cells according to the markings on the stem which is divided into 10 units of 10 divisions each. Each division represents 1 per cent.

FIG 64 —VAN ALLEN HEMATOCRIT TUBE.

FIG 65 —SPRING SEALING CLIP

7. Centrifuge again for five minutes and make another reading. If the two readings are similar it is taken as the per cent of red cells to whole blood. If, however, the reading is lower than the first the tube is again centrifuged for five minutes. This is continued until two readings are identical. With a little experience one soon learns the time and speed required.

8. Read off the measure of cells and multiply by 2 to convert into the volume per cent.

Haden-Sanford Method.—1. Make venous puncture with a sterile needle and syringe and withdraw exactly 5 c.c. of blood.

2. Immediately transfer the blood to a tube carrying exactly 1 c.c. of 1.6 per cent solution of sodium oxalate. The Sanford tube is recommended. The

capacity is 6 c.c. so that the graduations in 0.1 c.c. are well separated and the level of the packed cells easily seen.

3. Mix well and centrifuge for forty-five minutes at about 2500 revolutions per minute or until maximal packing of cells has taken place.

4. Forty-eight per cent of cells will pack to the 2.4 c.c. mark which may be taken as 100 per cent. According to the following table the volumes may be converted into per cent:

Volume of Erythrocytes, c.c.	Per Cent	Volume of Erythrocytes, c.c.	Per Cent
2 40	100 0	1.30	54.0
2.35	98.0	1.25	52.0
2 30	96 0	1.20	50.0
2.25	94.0	1.15	48.0
2.20	91 5	1.10	45.8
2.15	89 0	1.05	43.7
2.10	87.5	1 00	41.5
2.05	85 0	0 95	39.5
2.00	83.3	0 90	37.5
1.95	81 0	0 85	35.5
1.90	79 0	0.80	33.0
1.85	77.0	0.75	31.0
1.80	75.0	0.70	29.0
1.75	73.0	0.65	27.0
1.70	70.8	0.60	25.0
1.65	68.6	0 55	23.0
1.60	66.6	0.50	20.8
1.55	64.5	0.45	18.4
1.50	62.5	0.40	16.3
1.45	60.5	0.35	14.3
1.40	58.0	0.30	13.2
1.35	56.0	0.25	10.4

5. Or mix the blood thoroughly and place in graduated centrifuge tube or in any straight tube in which the column of blood can be measured. The Sahli tube used in estimating hemoglobin is very handy for this purpose as the graduations can be used for reading the volume. Fill the tube to the 100 per cent mark.

6. Centrifuge and read as described under the finger method.

DETERMINING THE VOLUME INDEX OF BLOOD

Principles.—The volume index is really an indirect method for determining the size of erythrocytes. In pernicious anemia the cells are larger than normal; therefore the volume will be greater. The reverse is true of the secondary anemias. In acute loss of blood the cells are normal because of the fact that there has been no disturbance in their production but that they have been merely reduced in number.

Procedure.—1. Determine the per cent of cells by volume by either of the methods described above.

2. Make a red cell count. The result is expressed in percentage, taking 5,000,000 as the normal. *To convert the count into percentage multiply the first two numbers by 2.* For example, if the count has been 4,800,000, the per cent is 96.

3. Divide the per cent by volume by the per cent obtained by counting. The result is the *volume index*:

$$\frac{\text{per cent by volume}}{\text{per cent by count}} = \text{volume index}$$

4. The normal varies from 0.85 to 1. In pernicious anemia the index is more than 1.0; in secondary anemia less than 1.0.

ESTIMATING THE COLOR INDEX

1. Determine by dividing the percentage of hemoglobin by the percentage of red cells as compared with a recognized normal; namely, 5,000,000 red cells per 1 c.mm.

2. In routine work the color index may be determined by dividing the percentage of the hemoglobin obtained, by the first two figures of the red cell count multiplied by 2.

3. The normal varies from 0.85 to 1.0. In secondary anemia it is normal or reduced; in chlorosis it is reduced; in pernicious anemia it is increased.

DETERMINING THE SATURATION INDEX

1. Determine the percentage of hemoglobin.

2. Determine the volume per cent of packed erythrocytes.

3. Divide the hemoglobin by the volume per cent and the result is the saturation index. The same result is obtained by:

$$\frac{\text{color index}}{\text{volume index}} = \text{saturation index}$$

4. The normal range is from 0.8 to 1.0. In secondary anemia it is normal or reduced; in chlorosis it is reduced.

TOTAL LEUKOCYTE COUNTING

Principles.—1. Blood is accurately diluted 1:20 with a fluid producing complete hemolysis of erythrocytes but without injury to the leukocytes. The corpuscles contained in 1 c.mm. of the diluted blood are then counted in a special chamber and this number multiplied by 20 (the dilution factor) to obtain the number in 1 c.mm. of undiluted blood. This constitutes the method for reporting the results.

2. The normal number of leukocytes is usually stated to be from 5000 to

10,000 per c.mm.; however, normal counts may be slightly lower or higher according to age, influence of digestion and *the time of day*. There is a greater normal fluctuation in the number of leukocytes than in the erythrocytes. In the morning the number is usually the lowest and gradually rises until evening, probably as a result of exercise. There may be as much as a 100 per cent increase but still the count may fall within the normal range. It is therefore advisable to *record the time of day a leukocyte count is made.* A count made during the afternoon may be 2000 higher than one made in the morning. Such an increase may not be necessarily due to disease.

3. This normal fluctuation is due to a constant change in the number of neutrophils from the bone marrow. The lymphocytes remain more constant with but a slight fluctuation without any definite tendency to an evening rise.

4. In disease the total number of leukocytes may increase; this is called *hyperleukocytosis* or *leukocytosis*. Or they may be decreased, called *hypoleukocytosis* or *leukopenia*.

5. In itself the total leukocyte count is of limited value for either diagnostic or prognostic purposes. It is, however, extremely valuable when used with the differential count to determine increases and decreases of the various types of leukocytes.

Procedure.—1. Draw blood to 0.5 of the Thoma pipet marked 11, or fill the stem of the Trenner pipet, as described in the method for counting erythrocytes.

2. Draw up diluting fluid to mark 11, thus making a dilution of 1 : 20.

DILUTING FLUID FOR LEUKOCYTES

Acetic acid............................ 3 to 5 c.c.
Water................................ 100 c.c.

Add a few drops of an aqueous solution of gentian violet or methylene blue to slightly stain the leukocytes.

3. Rotate the pipet well for several minutes, holding it in a horizontal position; finally shake sideways.

4. Blow out several drops.

5. Fill the counting chamber in exactly the same manner as described for the counting of erythrocytes.

6. Allow the cells to settle for at least three minutes.

7. Center the light and focus exactly as described for the erythrocyte count.

8. If the Levy or Levy-Hausser chamber is used, the cells in the four corner sq. mm. areas are counted in both rulings of the double chamber, also the cells in both central sq. mm. areas, *i.e.*, the cells in ten sq. mm. areas in all. This count is multiplied by 20 (dilution factor), which gives the total leukocytes per c.mm. of undiluted blood. *Or count the cells in the four corner squares and multiply by 50.*

9. When the cells are counted in one ruling only, the total count of the five

sq. mm. areas, as above, will represent the number of leukocytes in 5 sq. mm. or ½ c.mm. and must be multiplied by 40.

10. If other rulings are used count the number of cells per sq. mm. (the entire ruled-off area) and multiply by 200.

11. The same errors as may occur in erythrocyte counting must be kept in mind and carefully avoided (see page 72).

METHODS FOR STAINING BLOOD SMEARS

Principles.—1. An examination of the erythrocytes and different kinds of leukocytes possesses a great deal of diagnostic value. For this purpose stained preparations are superior to unstained or wet preparations.

2. The blood must be prepared in thin, evenly distributed smears on slides or cover glasses as described on page 60. *Well prepared films or smears are absolutely essential for accurate results.* For routine work a variety of staining methods are available. Preference should be given the polychrome stains, that is, those capable of staining the neutral, acid and basic granules of leukocytes. Poorly prepared and faultily stained smears cannot give results of acceptable accuracy.

Hematoxylin and Eosin Method.—1. Fix the smear by placing in methyl alcohol for five minutes.

2. Dry in the air.

3. Stain with Delafield's hematoxylin for three minutes; wash with tap water.

4. Stain with 1 per cent eosin for one minute; wash with tap water.

5. Dry thoroughly (gentle warming may be used) and examine; if cover glasses are used, mount on slides with balsam.

6. This is not a polychrome stain and does not stain the basic granules of basophilic leukocytes (granules appear as vacuoles in the cytoplasm).

Wright's Method.—1. Cover smear with 8 drops of Wright's stain[2] and allow to remain for two minutes.

2. Add to the stain on the smear an equal amount of distilled water and allow the stain thus diluted to stand two minutes more (the stain should now have a "brassy" sheen).

3. Wash with tap water and stand on end to dry.

4. Dry and mount in balsam if cover glass is used.

5. This is an excellent polychrome stain and is recommended for routine work.

6. The following method, employing a buffer solution, is also recommended by Giordano:

(*a*) Buffer solution: 6.63 grams of acid potassium phosphate (KH_2PO_4) and 3.2 grams of dibasic sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) are dissolved in 1000 c.c. of distilled water.

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[2] Wright's, Jenner's and Giemsa's stains may be purchased from supply houses. It is cheaper to purchase the powders (National Aniline Company or Coleman and Bell) and dissolve 0.3 gram in 100 c.c. of special acetone-free methyl alcohol.

(*b*) Add 25 to 30 drops of Wright's stain to blood film and allow to stand one minute. Add an equal number of drops of buffer solution and allow to remain four minutes. Wash with distilled water and dry.

7. Feemster recommends the following method:

(*a*) Solution No. 1: Place enough dry stain in a clean dry bottle to saturate absolute methyl alcohol (not more than 0.3 gram will dissolve in 100 c c.). Allow to stand for one or two days with occasional shaking. Filter and add one-fifth of its volume of methyl alcohol.

(*b*) Solution No. 2: In a second bottle place 90 c.c. of 95 per cent ethyl alcohol and 10 c.c. of distilled water. Add 0.3 gram of dry stain; mix and stand for two days with occasional shaking. Filter before use.

(*c*) Cover the dried blood smear with solution No. 1; *drain off excess at once* and let stand until the slide *turns red*.

(*d*) Flood with distilled water; let stand for one or two minutes.

(*e*) Wash with solution No. 2 until most of the red precipitate disappears; that over the smear usually disappears immediately. Wipe off ends of slide.

(*f*) Wash with distilled water, dry and examine.

Jenner's Method.—1. Cover smear with Jenner's stain[3] for three to five minutes.

2. Wash carefully with water until lavender color.

3. Dry; mount in balsam if cover glass is used.

4. This is a polychrome stain and generally satisfactory.

Giemsa's Method.—1. Fix the smear in methyl alcohol for two to five minutes.

2. Submerge the slide in diluted Giemsa's stain[3] for twenty-five to thirty minutes:

Giemsa stain	1 c.c.
Water (distilled)	10 c.c.

The water should be neutral or very slightly alkaline. It is advisable to test it before use by placing a few grains of hematoxylin in about 5 c.c. In from one to five minutes a pale violet color should appear; if it does not, the water is unsuitable for use.

The stain should be used immediately after diluting.

3. Wash with distilled water and dry thoroughly.

4. This stain is recommended for the detection of malarial parasites and for special examinations but not for routine differential leukocyte counts.

Pappenheim's Pyronine-Methyl-Green Method.—1. Fix smears with heat.

2. Cover with the staining fluid for from one-half to five minutes.

Methyl green (sat. aq sol)	30 to 40 c.c.
Pyronine (sat aq. sol.)	10 to 15 c.c.

This mixture will keep for about one month. If it is found that one of the dyes stains too intensely it can be reduced by adding more of the other dye.

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[3] See Footnote 2.

3. Wash with distilled water and thoroughly dry.

4. This stain is for special purposes only as described below.

THE LEUKOCYTES OF NORMAL BLOOD; VARIOUS CLASSIFICATIONS

1. Three separate kinds are recognized, namely, (a) lymphocytes from lymphoid tissues; (b) monocytes from the reticulo-endothelial system and (c) granulocytes from bone marrow.

2. The last are so called because of granules in their protoplasm and are subdivided according to the staining reactions of these into three types: neutrophils, basophils and eosinophils.

3. *Lymphocytes* (Plate I, Figs. 13, 14, 15) vary in size from about that of an erythrocyte to that of a neutrophil. The nucleus is round and stains deeply with the basic stain. The smaller ones stain more deeply and have a small amount of cytoplasm. The larger ones often stain less intensely and have more cytoplasm, in some of which may be seen several round, reddish-purple azurophilic granules. Occasionally forms with indented nucleus appear. It is generally believed that the large, less deeply staining forms are the younger types which become smaller upon reaching maturity. An increase of these cells is called *lymphocytosis* and a decrease *lymphopenia*.

4. *Monocytes* (Plate I, Figs. 16, 17) include cells which were formerly called large monocytes and transitionals. They are sometimes known as endotheliocytes. They are the largest type of leukocyte found in the blood (14 to 20 micra). The nucleus is less deeply stained than that of the lymphocytes, is usually indented and at times is horseshoe shaped. The chromatin material in the nucleus has a skeinlike appearance. Those with round nuclei are often difficult to distinguish from lymphocytes. There is a wider band of cytoplasm than that of the lymphocytes. The lymphocytes are not usually as large as neutrophils, while the monocytes are usually larger. The chromatin of lymphocytes is more granular in appearance. An increase is called *monocytosis*.

5. *Neutrophils* (Plate I, Fig. 18; also Plate II, Cell 14) are easily recognized by an irregular-shaped and lobulated nucleus, for which reason they are commonly known as "polymorphonuclears." Their average size is about 12 micra. The nucleus may be ribbon, bandlike or segmented. The segments vary in number from one to six or seven and are all connected by narrow nuclear bands. The cytoplasm contains numerous fine granules which do not stain definitely either blue (basic) or red (acid) and hence are regarded as neutral or neutrophilic. They may undergo an increase, designated as "neutrophilia," or a decrease, called "neutropenia." They have been subdivided by Arneth, Schilling and others according to the number and shapes of nuclei (described below).

6. *Eosinophils* (Plate I, Fig. 19) are granulocytes similar to the neutrophils except for a difference in the size and staining properties of the granules,

PLATE I —BLOOD CELLS STAINED WITH A ROMANOWSKY STAIN.

(All drawings made with camera lucida; × 1200. Wilson's stain.
Modified Romanowsky.)

1. Normal red corpuscle.
2. Pale or anemic corpuscle.
3. Basophilic granules in erythrocyte.
4. Nuclear particle (Howell's body) in erythrocyte
5. Erythrocyte containing nuclear particle and basophilic granules.
6. Polychromatophilic red cell containing a Cabot's ring body.
7. Normoblast.
8. Slightly polychromatophilic erythrocyte containing a nuclear particle, Cabot's ring body, and violet colored basophilic granules.
9. Normoblast showing an early stage of karyorrhexis.
10. Megaloblast, markedly polychromatophilic
11. Poikilocyte, markedly polychromatophilic and exhibiting reddish basophilic granules.
12. Blood platelets.
13, 14. Small lymphocytes.
15. Large lymphocyte, exhibiting azurophilic granules.
16 Large mononuclear leukocyte with a few azurophilic granules in the cytoplasm.
17. "Transitional" leukocyte with fine azurophilic granules.
18. Polynuclear neutrophilic leukocyte
19. Polynuclear eosinophilic leukocyte
20. Mast cell or polynuclear basophilic leukocyte.
21. Neutrophilic myelocyte.

PLATE I

R.S.M.
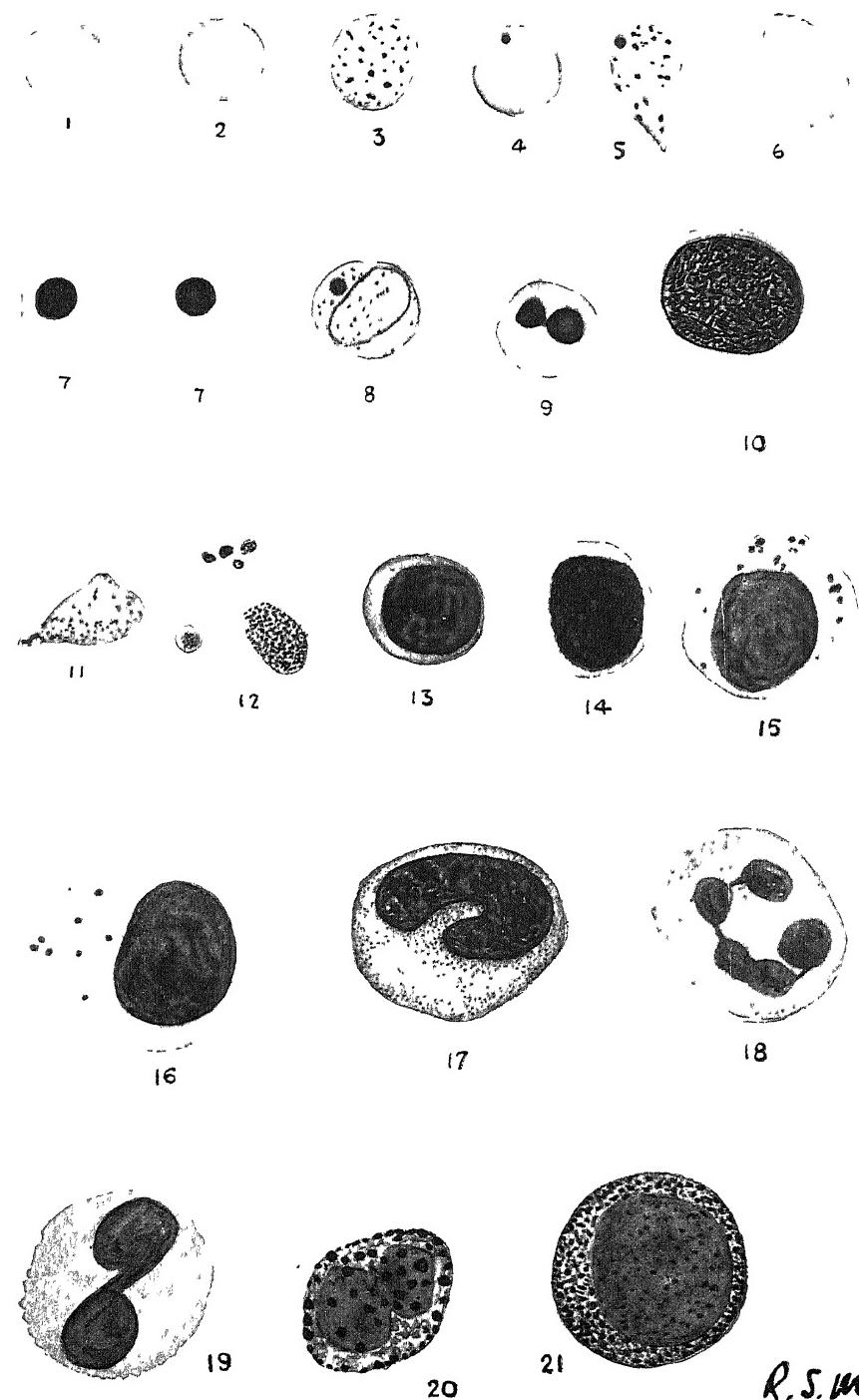
which are round or oval and large enough to be distinctly outlined. They stain pink to bright red (acid stain) with Wright's stain. An increase is called "eosinophilia" and a decrease "eosinopenia."

7. *Basophils* (Plate I, Fig. 20) are granulocytes similar to the neutrophils except that they contain granules which are larger and stain deep purple (basic stain) with Wright's stain. The nucleus is usually without distinct lobulation. The cell itself is slightly smaller than the neutrophil. They are also called "mast cells." An increase is called "basophilia" or "basophilic leukocytosis."

Neutrophils.—ARNETH'S CLASSIFICATION.—The youngest or most immature type of the neutrophil has a single round or indented nucleus; the older or more mature forms have *two* or more variously shaped nuclei. The youngest forms are but occasionally found in normal blood but in acute infections these types undergo an increase. Therefore it is of diagnostic and prognostic importance to subdivide these cells according to the nuclei as first suggested by Arneth, who divided them into five classes as follows:

Class	*Per Cent*
I. One round or indented nucleus..........	5
II. Two-lobed nucleus	35
III. Three-lobed nucleus	41
IV. Four-lobed nucleus	17
V. Five or more lobes....................	2

Each of these classes was subdivided according to the shape of the nucleus or nuclear divisions but only the subdivisions of Class I need be here mentioned from the standpoint of practical value:

The *M* cell with round nucleus
The *W* cell with a slightly indented nucleus
The *T* cell with a deeply indented nucleus

The *M* cell closely corresponds to the myelocyte and is not found in normal blood; the *W* and *T* cells correspond to the metamyelocytes and occur in 0.2 and 5 per cent respectively in normal blood.

In health the percentages of these various cells remain fairly constant but in disease a marked change may occur with or without a change in the total leukocyte count.

When there is an increase in Classes I and II (the younger forms) and a decrease in Classes IV and V it is called a *shift to the left*. When the opposite occurs it is called a *shift to the right*. A "shift to the left" is most marked in the severe acute infections and is considered an unfavorable sign, whereas a "shift to the right" is considered a favorable sign. When a shift to the left has occurred followed by shifting to the right the prognosis is considered favorable.

PAPPENHEIM'S CLASSIFICATION.—Pappenheim modified Arneth's classification by recommending the use of but three groups, namely, the myelocytes, metamyelocytes and mature forms. This classification is more practical than Arneth's due to the fact that a shift at one end is always at the expense of the other and furthermore, changes occurring at the left (increase or decrease of immature forms) are of most practical importance.

SCHILLING'S CLASSIFICATION.—Schilling has recommended a division of the metamyelocytes into two types, namely, (a) young forms with a round or slightly indented nucleus corresponding to Arneth's M and W cells, and (b) band forms with deeply indented or bandlike nuclei which correspond to Arneth's T cells (Fig 66). He has suggested a method for determining shifts to the left by the per cent of the various immature cells in the usual differential
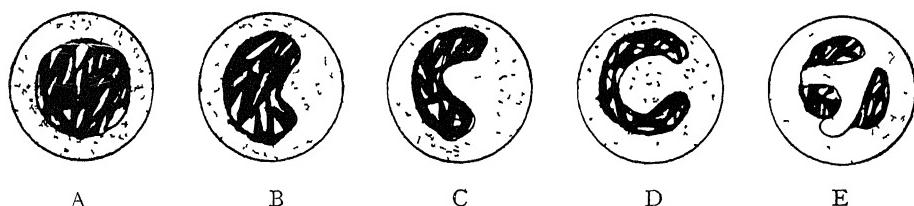
FIG 66 —SCHILLINGS' DIVISION OF NEUTROPHILS

A A myelocyte   *B* A young metamyelocyte   *C* and *D* Older, or banded, metamyelocytes   *E* Mature neutrophil.

count   By this method an increase or decrease of lymphocytes or other cells will influence the results.

ABNORMAL VARIETIES OF LEUKOCYTES

1. *Metamyelocytes,* mentioned above, are the connecting links between the myelocytes of the bone marrow and the polymorphonuclear neutrophils of the blood. The nucleus is indented (Fig. 66) and the cell usually smaller than the myeloblast and myelocyte, being about the size of the mature polymorphonuclear, which it resembles in every way except in the shape of the nucleus. These cells may be divided into two groups, namely, (a) those with a slightly indented nucleus called young or *Y* types and (b) those with a deeply indented nucleus called band or *B* type. The band form, although considered here as an immature type, is really the youngest mature type which is normally present in the blood   It is present in the proportion of about one band form to every fifteen or more mature forms (2 or more distinct nuclear segments).

2. *Neutrophilic myelocytes* (Plate I, Fig. 21; also Plate II, Cell 2) are the bone-marrow progenitors of the polymorphonuclear neutrophil of normal blood. *Eosinophilic myelocytes* and *basophilic myelocytes* are the bone-marrow progenitors of the eosinophil and basophil respectively of normal blood. All three, especially neutrophilic myelocytes, may be encountered in severe infec-

tions with extensive leukocytosis, but they are seen in large numbers especially in splenomyelogenous leukemia. All three are in turn derived from the myeloblasts of the bone marrow. All are large, mononuclear cells whose protoplasm is filled with neutrophilic, eosinophilic or basophilic granules. The nucleus occupies about one-half of the cell and is round, oval or slightly indented. The granules do not ordinarily stain as deeply as in the neutrophils, eosinophils, and basophils of normal blood. Rarely myelocytes are seen with both basophilic and eosinophilic granules. The basophilic myelocyte is usually smaller than the other two and its nucleus so pale and obscured with granules that it is not easily distinguished from the basophil of normal blood

3. *Myeloblasts* are the parent cells of the myelocytes and are encountered in acute myelogenous leukemia and the terminal stages of chronic myelogenous leukemia when the bone marrow reverts to the embryonic type. They do not have granules (Plate II, Cell 1). The nuclei are round or oval and poor in chromatin with pale blue nucleoli.

4. *Lymphoblasts* occur in acute lymphatic leukemia. They are large cells closely resembling the large or immature lymphocytes of normal blood, especially of children (Plate II, Cells 5, 6, 8, 9, 10, 11). Azure granules are sometimes seen in the cytoplasm, and the nucleus generally shows one or two nucleoli. At times the nucleus is lobulated and termed the "Rieder cell." The nucleus may also be lobulated in myeloblasts.

5. *Turck's irritation leukocytes* are of uncertain nature and diagnostic importance. By some they are regarded as pathological myeloblasts. They may be found in diseases associated with the irritation of the bone marrow (primary and secondary anemias, leukemias, malaria and pneumonia). They are large, mononuclear, nongranular cells with dense, opaque, strong basophilic cytoplasm which often contains vacuoles.

FIG 67—DOHLE INCLUSION BODIES. × 1500 (Wood)

6. *Plasma cells* are extremely rare in the blood and have no diagnostic significance. They resemble the Turck cell but may be differentiated by the presence of one or two nucleoli and especially by a tendency toward a radial or "wheel-like" arrangement of its chromatin.

7. *Degenerated leukocytes* (Plate II, Cell 3) are sometimes seen but have no significance unless present in large numbers. They occur as vacuolated leukocytes in the anemias and leukemias and as bare nuclei from ruptured cells.

8. *Dohle bodies* are *"inclusion bodies"* which are found in the cytoplasm of the neutrophils in certain diseases and particularly in scarlet fever. Although they may be present in a few cells in other diseases, their consistent presence in many of the cells in scarlet fever renders them of some diagnostic value in

PLATE II.—ABNORMAL TYPES OF LEUKOCYTES.*

Cell 1. Myeloblast from the blood of a case of chronic myelogenous leukemia. Wright's stain (Magnification † Zeiss apochrom. obj. 1.5 mm., compens. oc. No. 6). Note the delicate chromatin network and the two nucleoli. The nucleus shows an indentation opposite which the cytoplasm is less basophilic. There are no granules in the c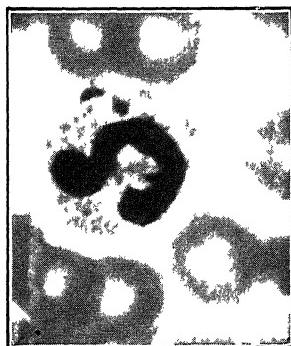ytoplasm. (From H. Downey, "The Myeloblast: Its Occurrence under Normal and Pathological Conditions and Its Relation to Lymphocytes and Other Blood Cells," *Folia Haematologica,* XXXIV, 1927, 34: 145–187, cell 46, Tafel 1.)

Cell. 2. Early myelocyte from dry smear of human marrow. May-Giemsa. (Magnification Zeiss apochrom. obj. 2 mm., compens. oc. No. 8.) Note the wide cell body and the finely granular structure of the nucleus which still shows nucleoli. The cytoplasm is still basophilic but contains a number of azure granules. This cell (No. 10 of Downey's plate in the article referred to) is classified by Downey as a myeloblast. He states on page 157 that "Naegeli would probably interpret these granules as immature neutrophile granules and so classify these cells as promyelocytes or leukoblasts" According to the criteria used in the classification adopted for this chapter the cell should be designated as immature myelocyte, perhaps of Class B. (From *ibid.,* cell 10, Tafel 1.)

Cell 3. Degenerated polymorphonuclear neutrophile from normal human blood. Wright's stain. The magnification is given by the adjacent red cell. This is a very characteristic form. (From F. R. Sabin and C. A. Doan, "The Presence of Desquamated Endothelial Cells, the So-Called Clasmatocytes, in Normal Mammalian Blood," *J. Exper. M.,* 1926, 43:823–827, cell 13, plate 32.)

Cell 4. Large clasmatocyte from the blood of a rabbit who had been given an intravenous injection of bovine tubercle bacilli. Wright's stain. Magnification given by the adjacent red cell. Note the densely compact granular character of the nucleus and the thin veil-like cytoplasm. (From *ibid.,* cell 12, plate 32.)

Cell 5. Very immature lymphocyte (lymphoblast) from the blood of a case of acute lymphatic leukemia. Wright's stain. (Magnification Zeiss apochrom. obj. 2 mm., compens. oc. No. 8.) The nucleus contains numerous nucleoli and its structure approaches that of the myeloblast. (Taken from Downey, *op. cit.,* cell 32, Tafel 1.)

Cell 6. Large lymphocyte with magenta (azure) granules. Wright's stain. Magnification shown by adjacent red cell. Note the pale blue cytoplasm and the dense nucleus which contains a nucleolus. (From G. R. Minot and L. W. Smith, "The Blood in Tetrachlorethane Poisoning," *Arch. Int. Med.,* 1921, 687–702, cell from plate opposite p. 690.) Size given by adjacent red cell.

Cell 7. Small lymphocyte. Wright's stain. Magnification shown by adjacent red cell. (From *ibid.*) Size given by adjacent red cell.

Cell 8. Atypical immature lymphocyte from a case of acute lymphatic leukemia. Compare with cell 5. (Magnification Zeiss apochrom. obj. 2 mm., compens. oc. No. 8.) (From H. Downey and C. A. McKinlay, "Acute Lymphadenosis Compared with Acute Lymphatic Leukemia," *Arch. Int. Med.,* 1923, 32: 82–112, cell 14, plate opposite page 101.)

Cells 9, 10, 11. Atypical lymphocytes seen in glandular fever. See text. (From C. W. Bladridge, F. J. Rohner and G. H. Hansmann, "Glandular Fever (Infectious Mononucleosis)," *Arch. Int. Med.,* 1926, 38: 413–448, cells from plate opposite p. 416.)

Cell 12. Monocyte from blood of a case of tetrachlorethane poisoning. Wright's stain. Magnification shown by adjacent red cell. Note the numerous azure granules in the cytoplasm, and an indented, polymorphous nucleus. (From G. R. Minot and L. W. Smith, *loc. cit.*)

Cell 13. Atypical monocyte from blood of a case of tetrachlorethane poisoning. Wright's stain. Magnification shown by adjacent red cell. Note the eccentric, indented nucleus with nucleoli and the cloudy cytoplasm with large numbers of fine carmine granules. This is perhaps a slightly stimulated form. (From *ibid.*)

Cell 14. Mature polymorphonuclear neutrophile from blood of same case as that from which cells 12 and 13 were taken. (From *ibid.*)

Cell 15. Megakaryocyte nucleus from the blood of a case of erythremia. Wright's stain. Magnification shown by adjacent red cell. Note the attached platelets. The nucleus of this cell should be compared with that of cell 4, a clasmatocyte. The two cells are frequently confused but their nuclei are wholly different. (From G. R. Minot, "Megakaryocytes in the Peripheral Circulation," *J. Exper. M.,* 1922, 36: 1–7, cell 6, plate 1.) Magnification indicated by adjacent red cell.

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* A collection of cells from various publications indicated in the legend. Romanowsky staining. Redrawn by Miss Grace MacMullen, under the direction of Mr. Louis Schmidt, Rockefeller Institute, New York City.

† The magnification indicated in the following text should be reduced one-fourth.

PLATE II

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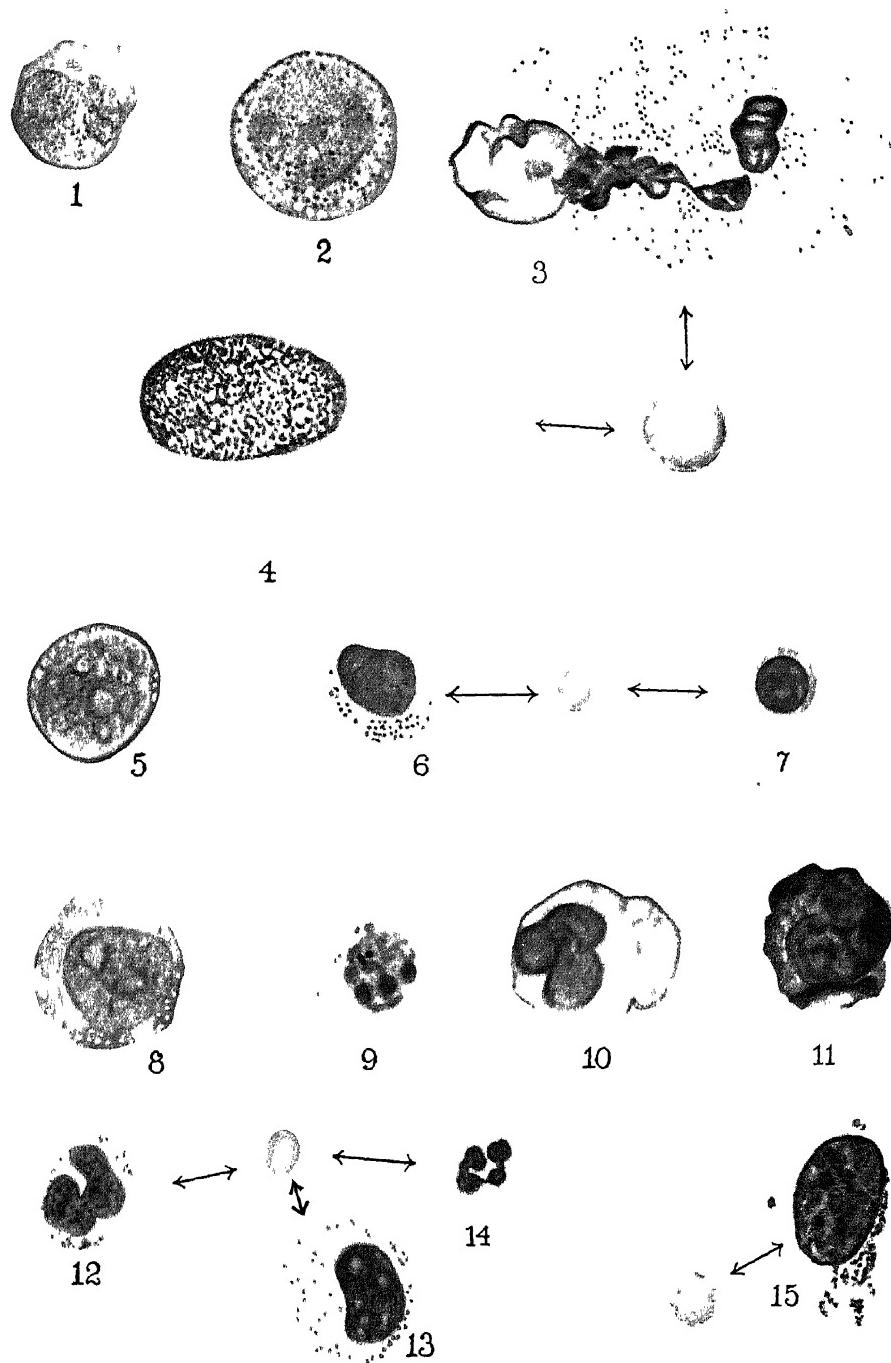
this disease. They are about the size of cocci, single or in pairs and some are pear-shaped (Fig. 67). Although they can be seen in smears stained with Wright's stain, a better method for demonstrating them is to stain with Pappenheim's pyronine-methyl green (see above), when they will occur as bright red with the nuclei of the cells blue to reddish purple.

DIFFERENTIAL LEUKOCYTE COUNTING

Principles.—1. Differential leukocyte counts refer to the actual number or percentage of different kinds present as variations from the normal, and possess a great deal of diagnostic and prognostic value.

2. The number of cells to be counted and classified should be determined by the total leukocyte count. In routine work the following is recommended:

> For total counts under 5000, classify 50 cells.
> For total counts of 5 to 10,000, classify 100 cells.
> For total counts of 10 to 15,000, classify 200 cells.
> For total counts of 15 to 20,000, classify 300 cells.
> For total counts of 20 to 25,000, classify 400 cells.
> For total counts over 25,000, classify 500 cells.

3. It is usual to submit a report in terms of the per cent of each type of leukocyte in the count, but a much better plan is to report the actual number of each leukocyte per c.mm. of blood which may be calculated from the total leukocyte count. If, for example, the total leukocyte count is 12,500 with 77 per cent neutrophils, the actual number of these cells per c.mm. of blood would be 125 × 77 or 9625.

4. According to the percentage system, the normal for adults is approximately as follows:

	Per Cent
Neutrophils	50 to 70
Basophils	0.5 to 1
Eosinophils	1 to 4
Lymphocytes	20 to 40
Monocytes	4 to 8

5. According to the actual number per c.mm. of blood, the normal for adults is approximately as follows (total leukocytes 5000 to 10,000):

	Per C.Mm.
Neutrophils	3000 to 7000
Basophils	0 to 50
Eosinophils	50 to 400
Lymphocytes	1000 to 3000
Monocytes	100 to 600

6. For children the figures are approximately as follows.

Leukocyte Cells	Three Months to Three Years	Three to Five Years	Over Five Years
Neutrophils	2000 to 7000	3000 to 8000	3000 to 7000
Basophils	0 to 50	0 to 50	0 to 50
Eosinophils	25 to 700	50 to 700	50 to 400
Lymphocytes	4000 to 9000	2500 to 6000	1000 to 3000
Monocytes	25 to 700	25 to 700	100 to 600

7. As the percentages do not indicate the actual number, it is advisable and recommended to convert them into the numbers of each type per c.mm. of blood, although the medical profession as a whole is not yet acquainted with the normal figures given above with which to interpret reports. *The per cent of any type of cell may be increased or decreased without any change in the actual number* by a change in the total leukocyte count.

8. When the per cent of a cell is higher than normal without any indications of an absolute increase as determined by the total count, it is spoken of as a "relative increase." But this term, which is so generally used, should be abandoned as it does not properly express any definite change. For example, a "relative lymphocytosis" may be due to a diminution in the number of neutrophils without any change in the normal number of lymphocytes; or the number of lymphocytes may be actually increased.

9. For simplicity the following nomenclature is advocated:

Neutropenia: decrease in polymorphonuclears

Basopenia: decrease in basophils

Eosinopenia: decrease in eosinophils

USUAL CHANGES IN

Neutrophilia	Neutropenia	Lymphocytosis
Severe acute infections, particularly those due to cocci	Subacute and chronic infections	Convalescence from acute coccus infections
Acute loss of blood	Common in diseases due to bacilli	Common to bacillary infections
		Syphilis
	Splenic anemia	Measles
	Agranulocytosis (malignant leukopenia)	Lymphatic leukemia
	Convalescence from severe acute infections	Chronic infections

Lymphopenia: decrease in lymphocytes
Monopenia: decrease in monocytes
Neutrophilia: increase in polymorphonuclears
Basophilia: increase in basophils
Eosinophilia: increase in eosinophils
Lymphocytosis: increase in lymphocytes
Monocytosis: increase in monocytes

10. The table gives the usual changes in the leukocytes in disease

Procedure.—1. Prepare several blood smears as described on page 60.

2 Stain with Wright's or other suitable stain (see page 77)

3. Examine with low-power lens to determine if the leukocytes are well distributed Look particularly at the edges and end of the smear. If they are not properly distributed, examine another smear.

4. If the slide proves satisfactory, systematically examine with oil-immersion lens by recording each type of leukocyte seen as the slide is moved from one field to another. The Marbel blood cell calculator (Fig. 42) is very convenient.

5. At the same time a special differential count may be made of the neutrophils for the forms of metamyelocytes and for determining the nuclear index (see below) to ascertain if there is any "shift to the left."

6 The red blood corpuscles should also be examined and any abnormalities noted with special reference to the number of nucleated cells seen during the count.

7 At least three separate parts of the slide should be examined.

LEUKOCYTES IN DISEASE

Lymphopenia	Eosinophilia	Eosinopenia	Monocytosis
Severe acute infections, particularly those due to cocci	Convalescence from acute infections, particularly those due to cocci	Severe acute infections, particularly those due to cocci	Acute infectious mononucleosis
Common in agranulocytosis (malignant leukopenia)	Parasitic diseases		Typhoid fever
	Bronchial asthma		Hodgkin's disease
	Eosinophilic leukemia		Protozoan diseases
	Scarlet fever		

8. It is possible to make a "rough" differential count, insofar as estimating the percentage of polymorphonuclears and lymphocytes is concerned, while doing the total leukocyte count by using the following diluting fluid (Nicholson) in the pipet: 0.6 c.c. of a 6 per cent Giemsa stain added to 10 c.c. of a 20 per cent solution of acetone in distilled water; make up daily. Make the count with a high dry lens with full light and the *condenser slightly down.*

9. It is recommended to make a separate count of the immature neutrophils or metamyelocytes and to include it routinely in all differential counts of bloods showing a leukocytosis due to acute infections. The *normal* average for an adult is approximately as follows (total count 5000 to 8000):

Young metamyelocytes..........	0 to 80 (0 to 1 per cent)
Old or banded metamyelocytes...	150 to 400 (3 to 5 per cent)
Polymorphonuclear neutrophils..	2550 to 5360 (50 to 67 per cent)
Basophils.....................	0 to 80 (0 to 1 per cent)
Eosinophils...................	100 to 320 (2 to 4 per cent)
Lymphocytes..................	1050 to 2800 (21 to 35 per cent)
Monocytes....................	200 to 640 (4 to 8 per cent)

10. As an example of a "shift to the left" or an increase of metamyelocytes, the following count from a case of acute suppurative appendicitis in an adult with a total leukocyte count of 15,000 may be given:

Young metamyelocytes................	350 (2.3 per cent)
Old or banded metamyelocytes..........	1700 (11.3 per cent)
Polymorphonuclear neutrophils..........	10,000 (69.7 per cent)
Basophils...........................	10 (0.06 per cent)
Eosinophils..........................	0
Lymphocytes........................	2000 (13.2 per cent)
Monocytes..........................	500 (3.3 per cent)

DETERMINING THE NUCLEAR INDEX

The "shift to the left" may be indicated by the *nuclear index* (Boerner) calculated as follows:

1. Make a differential count of a total of 100 myelocytes and mature neutrophils. Divide the number of mature neutrophils by the total of immature cells (myelocytes and metamyelocytes):

$$\frac{\text{total of mature}}{\text{total of immature}} = \text{nuclear index}$$

2. The normal index for an adult is 15 or over:

An index of 10 to 15 = very slight shift to left
An index of 5 to 10 = slight shift to left
An index of under 5 = marked shift to left

DETERMINING THE LEUKOCYTIC INDEX

1. The leukocytic index of a blood may be determined as follows:

$$\frac{\text{percentage of neutrophils}}{\text{percentage of lymphocytes}} = \text{leukocytic index}$$

2. It possesses little or no practical value.

DETERMINING THE ARNETH INDEX

1. The Arneth index may be computed as follows: Classify 100 neutrophils according to Arneth's five classes (see page 81). Add the cells of Classes I, II and one-half of those in III. This gives the index.

2. The normal range is from 51 to 65, with an average of 60. Shifts to the left increase the number and shifts to the right decrease it.

PEROXIDASE REACTION

Principles.—This test is employed for the identification of myeloblasts in the differential diagnosis of the leukemias. It is based upon the principle that a study of the oxidizing ferments of leukocytes aids in differentiating those of myeloblastic origin (myeloblasts) from those of other lineage. Owing to difficulties in staining, a large number of methods have been advocated.

Washburn's Method.—1. Thin smears should be made, allowed to dry and stained within three to four hours.

2. Flood the smear with 10 drops of solution No. 1 and allow to stand for one to one and one-half minutes.

SOLUTION NO. I

Benzidine base............................	0.3 gm.
Basic fuchsin..............................	0.3 gm.
Sodium nitroprusside (sat. aq. sol.)..........	1.00 c.c.
Ethyl alcohol (95 per cent).................	100 c.c.

Dissolve the benzidine and fuchsin in the alcohol in order named. Then add the nitroprusside solution. A slight precipitate may form at the bottom of the flask but does not interfere with the staining qualities. This solution will keep for eight to ten months.

3. Add 5 drops of solution No. 2 without pouring off No. 1 and allow to stand three to four minutes.

SOLUTION NO. 2

Hydrogen peroxide...................	5 to 6 drops
Tap water..........................	25 c.c.

This solution will keep for about two days.

4 Wash thoroughly with tap water (one-half to one minute).

5. While still wet, flood with 95 per cent ethyl alcohol and allow to stand three to four minutes, or until completely decolorized (*i e*, when there is no more pink visible to the naked eye).

6 Wash thoroughly with tap water and dry.

7. Flood with 8 drops of Wright's stain and allow to stand for two to three minutes.

8. Add 14 drops of tap water (one and one-half times as much water as Wright's stain) and allow to stand for twenty to forty-five minutes. Most normal and many abnormal bloods will stain well in twenty to twenty-five minutes but certain abnormal bloods, particularly the leukemic bloods, require thirty-five to forty minutes.

9. Wash briefly with tap water, flood with 95 per cent alcohol for three to five seconds and immediately wash with tap water for ten to fifteen seconds.

10. Dry and examine.

11. The *normal polymorphonuclear neutrophil* is so full of large black peroxidase granules that frequently the characteristic pleomorphic purple nucleus is the only other element visible. In the few polymorphonuclears which show a smaller number of black granules, the cytoplasm may be made out as finely granular and either neutrophilic or slightly pinkish. In certain abnormal bloods, notably in the myelogenous leukemias, some of these cells may have very few or no peroxidase granules. The cytoplasm of these cells frequently appears to be vacuolated, suggesting that they are degenerated forms in which the substance producing the peroxidase reaction has already disappeared.

12. The nuclei of *eosinophils* stain the usual purple, as with Wright's stain, and the large eosinophilic granules take on the deep black of the peroxidase stain but remain refractile. This gives them the appearance of very large black granules whose center is slightly paler than the periphery and of a brownish tint.

13. The *basophils* are the only normal cells which are hard to classify with this stain, since the basophilic granules take on the black of the peroxidase reaction so that their differentiation from neutrophils is difficult. The granules are slightly larger and tend to be arranged more thickly at the cell edges. The nucleus is also to be distinguished as less pleomorphic and of a paler purple or even lavender color compared with that of the neutrophils.

14. The *lymphocytes* never show any peroxidase granules. Their characteristics by this staining method are identical with those seen in a Wright stain.

15. The *endothelial type of mononuclear cell* usually shows a scattering of black granules which tend to be present largely in groups. The remainder of the cell stains as with Wright's stain. That is, the nucleus is a slightly paler shade of purple than that of the neutrophils or lymphocytes while the cytoplasm shows a slate-gray homogeneous background with fine pinkish granu-

lations unevenly distributed. Occasionally cells with only one or two or even without any peroxidase granules are seen

16. The *large mononuclears* invariably show few to many peroxidase granules. The granules usually appear smaller than those in the neutrophils and more diffusely distributed throughout the cell than is the case with the endothelial type of mononuclear. The nucleus and cytoplasm stain as with a simple Wright's stain. That is, the nucleus is purple and the cytoplasm very finely granular, giving the impression of a homogeneous blue gray until one examines it closely enough to observe the presence of granulation. Occasionally these cells are so full of black granules that their cytoplasm is obscure.

17. *Myelocytes* usually show some peroxidase granules. The number may vary from 2 to 3 to a score or more, so that the cell is as full of granules as a mature neutrophil. The myelocytes which are less granular by Wright's stain show fewer peroxidase granules. In cases of acute myelogenous leukemia when the blood is flooded with myelocytes in all stages of maturity with eosinophilic, basophilic and neutrophilic granulation, the peroxidase granules may be less clearly defined than normally.

18. *Myeloblasts and lymphoblasts* never show any peroxidase granules so that their differentiation is no more possible with this stain than with any other, if a difference actually exists. Their appearance is identical with that seen in Wright's stain. However, it is worth remembering that some of the large primitive leukocytes with deep blue cytoplasms and large round purple nuclei may be very early myelocytes whose granules are too few and small to be seen with the ordinary Wright's stain. Such cells will show a few black granules with the peroxidase stain, thus indicating a myelogenous origin

NORMAL AND ABNORMAL ERYTHROCYTES

1. The erythrocytes of stained blood smears should also be examined and reported upon, especially in the anemias.

2. Normally they are about 7.5 micra in diameter (Plate I, Fig. 1; also Plate III, Group 7). In stained preparations distorted shapes may be seen due to mechanical distortion in preparing smears. In wet preparations they may occur in rouleaux formation and show some crenation.

FIG 68—THE ERYTHROCYTES IN SICKLE-CELL ANEMIA.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co)

3. In the anemias with diminished hemoglobin, and especially in chlorosis, the central pale area becomes larger and paler, constituting *achromia* (Plate I,
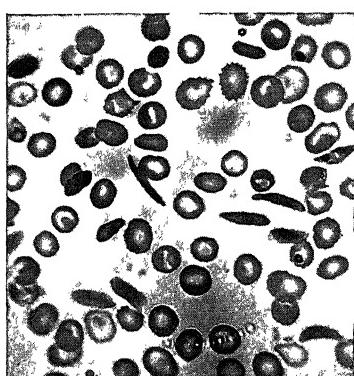
Fig. 2; also Plate III). In extreme instances the cells become mere rings ("pessary forms"). In pernicious anemia, however, many of the corpuscles stain deeply and entirely lack the pale center (Plate I, Fig. 5).

4. Abnormal variation in size is called *anisocytosis*. When smaller than normal the cells are called *microcytes* (Plate III) and when larger, *macrocytes* (Plate I, Fig. 13); extremely large forms are called *megalocytes*.

5. *Poikilocytes* are corpuscles with abnormal shapes (Plate III). They may be caudate, club-shaped, oval, elliptical or sickle-shaped. *Sickle-shaped* cells are especially numerous in a hereditary type of anemia more commonly seen in Negroes called "sickle-celled anemia" (Fig. 68).

When the blood is diluted with salt solution, placed on a slide, covered while wet and sealed with vaselin and kept in a warm place for eighteen to twenty-four hours, many typical sickle and crescentic forms will develop.

Elliptically shaped cells may occur in both races in various anemias also as a hereditary phenomenon. The above test is a useful aid in differentiation and in the diagnosis of sickle-cell anemia.

6. *Polychromatophilia* is the term used for indicating the abnormal affinity of erythrocytes for the basic stains (Plate III). When present, many erythrocytes will be seen taking the basic blue stain in varying degrees, usually pale to light blue instead of pale pink. The condition is abnormal and found in anemias where there is active regeneration of erythrocytes.

7. *Basophilic degeneration,* or *"stippling,"* is a condition in which there are many very fine to coarse blue dots or granules present in the erythrocyte (Plate I, Fig. 3). They are found in cases where erythrocytic regeneration is active and probably represent cells which have undergone a degenerative change before they were fully mature. Their presence in suspected cases of lead poisoning is of diagnostic value.

8. *Reticulated erythrocytes* are cells which, when stained with brilliant cresyl blue, show filaments which are well stained if the staining is done while the cell is still alive (vital staining). Although these filaments take the basic stain they will not stain in the usual dry smear. They are often arranged in skeins or wreaths.

9. *Nucleated red cells* are immature cells which are thrown into the circulation in severe anemias and leukemias in which there is an active regeneration of bone marrow. The *megaloblast* (Plate I, Fig. 10) is the largest type and has a large, oval, pale-staining nucleus. The cytoplasm often shows polychromatophilia. Some may closely resemble lymphocytes due to the blue staining of the cytoplasm. These cells are usually present in pernicious anemia, where their presence is of diagnostic value. The *normoblast* (Plate I, Fig. 9; also Plate III, Group 5) is of about the same size as an erythrocyte and has a nucleus more deeply stained than the megaloblast with an irregular arrangement of the chromatin. Occasionally the chromatin is arranged in a manner resembling the spokes of a wheel. They often show polychromatophilia. The younger forms may exceed 10 micra in diameter and be confused with the megaloblast.

The older forms are smaller and the nucleus deeply stained. There may be more than one nucleus or the nucleus may be irregular, lobulated or fragmented. If the nucleus is completely broken up the fragments may all disappear except for a few. These remaining particles are called *nuclear particles* or *Howell-Jolly bodies* (Plate I, Figs. 4, 5). The normoblast is probably not an older form of the megaloblast but a distinctly different cell. The smallest nucleated red is called a *microblast*. It measures less than 5 micra in diameter, has a deeply staining nucleus and is regarded as an older form of normoblast.

Cabot's ring bodies (Plate I, Fig. 6) are ring or figure-eight-shaped structures which stain red or reddish-purple with Wright's stain. They are seen in lead poisoning, pernicious anemia, leukemia and especially in erythroblastic anemia of children.

COUNTING RETICULATED CELLS

1. Place in a small test tube the following solutions and thoroughly mix:

Brilliant cresyl blue (Grubler) (sat. alc. sol.)................. 5 drops
Neutral potassium oxalate (1 per cent) in sodium chloride solution
  (0.85 per cent sol.) . ... 25 drops

2. Prick the finger and allow 2 or 3 drops of blood to fall into the stain.
3. Mix thoroughly and allow to stand thirty minutes.
4. Centrifuge; pour off the supernatant fluid; transfer a small drop to a glass slide and make a thin smear. Allow to dry without heat. The sediment of cells should be of about the same density as the blood. To obtain this, leave about as much by volume of the supernatant fluid as cells. Mix the cells thoroughly before smearing and make thin films so they will dry rapidly.
5. Stain with Wright's or other suitable stain.
6. Estimate the per cent of red cells that are reticulated. This can be done by examining with the oil-immersion lens and counting 2000 cells, noting the number of cells showing reticulum.

$$\frac{\text{number of reticulated cells}}{20} = \text{per cent}$$

Normal findings are below 1 per cent. An increase is considered one of the early signs of regeneration of red cells by the bone marrow.

COUNTING PLATELETS

Principles.—1. Blood platelets are stained by the polychrome dyes like those of Wright and Jenner and are spheric or ovoid, reddish to violet, granular bodies appearing as clumps in ordinary blood smears (Plate I, Fig. 12; also Plate III).

2. Because of clumping, special methods for counting have been devised; divided into those that compute their number in relation to the number of

PLATE III—NORMAL AND ABNORMAL ERYTHROCYTES

The preparations are all from cases of erythremia (polycythemia vera) except No 7 which is from a normal person   The stain is Wright's stain and the magnification is about 970   The drawings are made with the aid of a camera lucida and represent actual fields

Group 1   Shows especially macrocytosis   The nucleated red cell is an erythroblast

Group 2   Shows faint polychromatophilia and moderate anisocytosis   The flattening out of the cells is due to the increased viscosity of the polycythemic blood   Red cell count 10,000,000 per c mm

Group 3   Shows marked achromia, anisocytosis and polychromatophilia   The red cell count was 8,500 000 per c mm and the hemoglobin 105 per cent   Color index 0 6

Group 4   Shows marked anisocytosis, poikilocytosis and polychromatophilia   The white corpuscle shown is presumably a myeloblast   The nucleus contains several nucleoli   Below this cell is a red corpuscle containing a Howell-Jolly body   Red cell count 3 300,000 per c mm

Group 5   Shows numerous fragmented cells, microcytes, macrocytes and coarse stippling   One of the red cells shows a Cabot-ring body   A normoblast is shown

Group 6   Shows slight anisocytosis and fine stippling   The uniform gray color as compared with the cells of group 7 is due to a difference in the stain   In judging polychromatophilia the variation of the staining of individual cells in a given preparation and not the color of the preparation as a whole must be taken as a criterion

Group 7   Shows normal red cells

Note that in all the groups the number of platelets is normal or increased   (From Minot and Buckman, *Am J Med Sc*, 1923, 166 470)

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PLATE III
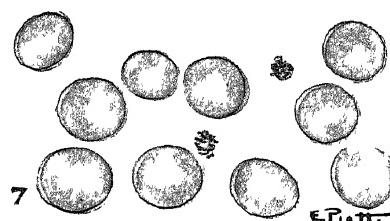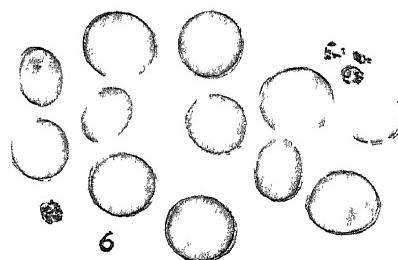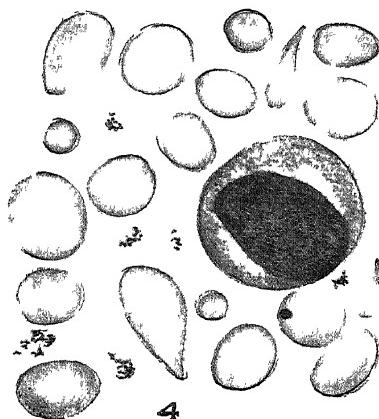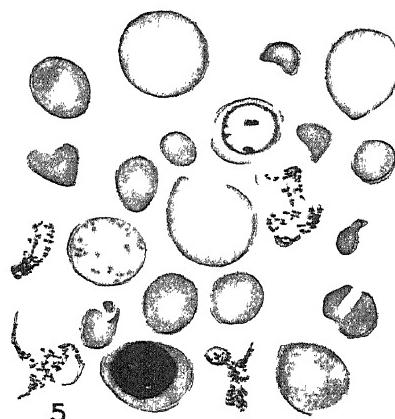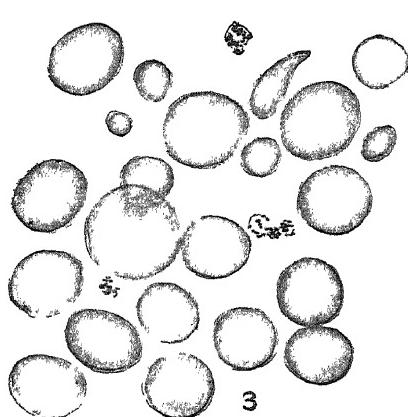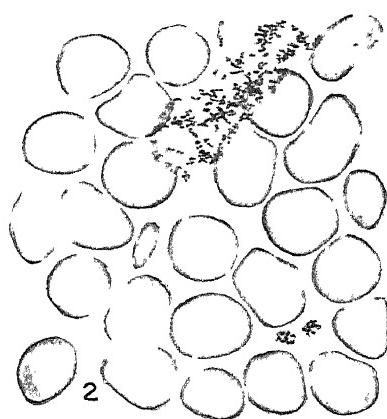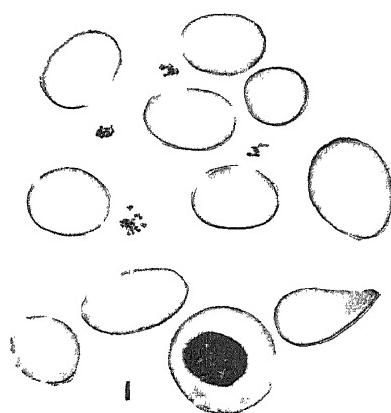
erythrocytes (indirect) and those that make a direct count with a diluting pipet.

3. The exact source of platelets is as yet unknown, but it is generally believed that they are detached portions of the cytoplasm of megakaryocytes of the bone marrow (J. H. Wright) and have an important rôle in the coagulation of the blood.

Fonio's Smear Method.—1. Prepare and puncture finger with lancet and immediately place a drop of 14 per cent magnesium sulphate over the puncture before the blood begins to flow.

2. With gentle pressure allow the blood to flow into the sulphate solution. When the proportion is about 1 of blood to 5 of sulphate, mix thoroughly.

3. Transfer a drop to a clean slide and make a thin smear in same manner as described for differential counts. Prepare several smears.

4. Wipe the finger clean and proceed to make a red cell count.

5. Stain the slide with Wright's blood stain, being sure that the stain is of proper reaction to stain the platelets well as otherwise they may be palely stained and difficult to count.

6. Cut a small square in a circular piece of paper and place it in the ocular of the microscope to reduce the size of the field.

7. Focus and count the number of erythrocytes and the number of platelets in the field. Continue to count fields over various parts of the slide (center and both ends) until 1000 erythrocytes have been counted.

8. The number of platelets counted to 1000 erythrocytes is multiplied by the number of thousands of erythrocytes as determined by the erythrocyte count.

Example: Patient's erythrocyte count is 4,500,000. The number of platelets counted to 1000 erythrocytes is 39. Dividing the total erythrocyte count by 1000 to determine the number of thousands of erythrocytes gives 4500. Multiplying 39 by 4500 gives 175,500 as the platelet count.

$$\text{number of platelets counted} \times \frac{\text{erythrocyte count}}{1000} = \text{platelet count}$$

9. The normal is from 200,000 to 400,000 per c.mm. of blood.

Direct Method.—1. Rapid work is necessary in order to prevent clumping of platelets.

2. With a red-corpuscle pipet draw one of the diluting fluids given below to near the 1 mark; then blood from a freely flowing puncture to exactly 0.5 and finally diluting fluid again to 101 (blood dilution of 1 : 200).

WRIGHT AND KINNICUTT'S DILUTING FLUID

Brilliant cresyl blue (aq. sol. 1 : 300) . . . . 2 parts
Potassium cyanide (aq. sol. 1 : 1400) . . . . 3 parts

Keep the solutions separately. The dye keeps indefinitely; the cyanide solution keeps about ten days. Mix and filter just before using.

REES AND ECKER'S DILUTING FLUID

Sodium citrate (3.8 per cent aq. sol.) .... 100.0 c.c.
Formalin .... 0.2 c.c.
Brilliant cresyl blue....................... 0.1 gm.

Filter before using.

LEAKE AND GUY'S DILUTING FLUID

Water (distilled)......................... 94.0 c.c.
Formalin............... .... 6.0 c.c.
Sodium oxalate............... ... . 1.6 gm.
Crystal violet....... .... 0.05 gm.

The fluid is warmed, filtered and kept in a bottle (keeps well).

3. Shake for two minutes.

4. Fill counting chamber as in making an erythrocyte count.

5. Allow to stand for ten minutes.

6. Examine with 10X ocular and 4 millimeter objective.

7. Count the platelets in 200 small squares and multiply by 4000 to obtain the number per c.mm. of blood.

EXAMINATION OF BLOOD FOR MALARIA

Principles.—1. The laboratory diagnosis of malaria is based entirely upon an examination of the blood for the plasmodia. Cultural and animal inoculation tests have not been successfully developed. The three principal varieties of plasmodia may be identified in blood smears and especially in those properly stained.

2. Wet or fresh smears are not recommended, although useful. Well-prepared thin smears properly stained with Wright's, Giemsa's or some other polychrome stain are recommended.

3. *Great care is required against regarding blood platelets (usually surrounded by a halo) accidentally placed on erythrocytes as being malarial parasites (no haloes).* Bacteria and dirt may be likewise sources of error, especially in thick smears. In chronic malaria the thick blood or some other concentration method may be required for the detection of parasites.

Fresh Wet Blood Method.—1. Use perfectly clean and grease-free cover glasses or slides.

2. Puncture the finger or lobe of the ear and take up a small drop of blood by touching same with center of a cover glass.

3. Place on slide so that the blood will spread out in a thin film.

4. If examination is prolonged, seal edges of cover glass with melted vaselin to prevent drying.

5. Examine at once with oil-immersion objective.

6. The best time for examination is six to eight hours after a paroxysm, but the parasites can be found at other times and the examination should be made without any special reference to the occurrence of paroxysms.

Thin Smear Method.—1. Smears are prepared as for differential leukocyte count but so thin that the red cells lie flat and well separated.

2. Fix and stain with Wright's or Giemsa's stain in same manner as staining for differential leukocyte counts.

3. Dry and examine with 1/12 oil-immersion objective.

4. The smears must be well stained for satisfactory results. Unless the nuclei of leukocytes are well stained and have the proper reddish tint due to proper staining of the chromatin, the chromatin of the plasmodia will not be properly stained. Good and poor areas may be on the same slide.

5. Malaria plasmodia are in or on the erythrocytes, and no object should be considered as a probable plasmodium unless it is so situated.

Identification of Plasmodia.—*Tertian* parasites (Plate IV) undergo asexual development in forty-eight hours; erythrocytes swollen and pale; young parasites pale and transparent; outline indistinct; active ameboid movement in wet preparations; mature parasites large and distending corpuscles; very fine, brown, scattered pigment granules, 24 to 32 segments; approximately round gametes; all sizes of schizonts.

Quartan parasites (Plate IV) undergo asexual development in seventy-two hours; erythrocytes reduced in size and darker than normal; young parasites waxy, highly refractive with distinct outlines; sluggish movement in wet preparations; mature parasites smaller than tertian; coarse, peripherally arranged pigment granules; 6 to 12 segments; approximately round gametes; all sizes of schizonts.

Estivo-autumnal parasites (Plate IV) undergo asexual development in about forty-eight hours; erythrocytes unchanged in size and color or darker; distinct outline of parasite with active movements in wet preparations (young forms only in peripheral blood); very few, minute, inactive, medium to coarse granules (distinctly pigmented forms seldom seen); segments rarely seen in peripheral blood; crescentic and oval gametes; schizonts only in rings.

Thick Smear Method of Barber and Komp.—1. It is essential to carefully clean the skin with alcohol and gauze in order that the blood be free of dirt, bacteria, dust or other débris. The slides should be perfectly clean.

2. Put on a drop three or four times as large as used for ordinary thin blood smears. Spread by dragging the drop on the surface of the slide with the sticking needle or corner of another slide.

3. The smears should be dried enough to make them adhere, but too much drying will prevent a clear staining of the parasites. In ordinary summer weather it is sufficient to keep the smears overnight in a box with closed lid;

(*A, B, C,* Wright's stain. *D,* Leishman's stain. *E,* Ziehl-Neelsen stain; × 800)

A. Plasmodium vivax (tertian). 1, a red cell with platelet superimposed; 2 to 4, hyalin (nonpigmented) parasites; 5 to 8, pigmented parasites; cell 5 shows Schüffner's granules in the red blood corpuscle; 9, daughter parasites or merozoites and free pigment; 10, macrogamete (the female sexual form); 11, microgametocyte (the male sexual form); 12, a large mononuclear leukocyte (macrophage) containing malarial pigment. Note swelling and pallor of infected red cells.

B. Plasmodium malariae (quartan). 1, a red cell with blood platelet superimposed; 2, 3, hyalin (nonpigmented) parasites; 4 to 8, pigmented parasites; 5 is a band form of the parasite; 9, daughter parasites or merozoites and free pigment; 10, macrogamete (the female form); 11, microgametocyte (the male sexual form). Note absence of swelling and pallor of infected red cells.

C. Plasmodium falciparum (syn.: *Laverania malariae*) (aestivo-autumnal, malignant tertian, subtertian). 1, a normal red cell; 2 and 4, hyalin (nonpigmented) parasites; 3, multiple infection of an erythrocyte; 5 to 8, pigmented parasites; 9, daughter parasites and free pigment. *The forms included within the brackets are rarely encountered in the peripheral blood.* Note premature division of the chromatin of the parasite. 10, macrogamete (the female sexual form); 11, microgametocyte (the male sexual form). Note the "apron" or "bib" on the crescents, 10 and 11. 12, a large mononuclear leukocyte (macrophage) containing malarial pigment.

D. Leishmania donovani (Leishman-Donovan bodies) in the large mononuclear leukocytes and free in the plasma (combined field).

E. Tubercle bacilli in the sputum. Upper half: unselected particle of sputum taken for staining. Lower half: same specimen; the material for staining was a particle containing elastic tissue.

PLATE IV
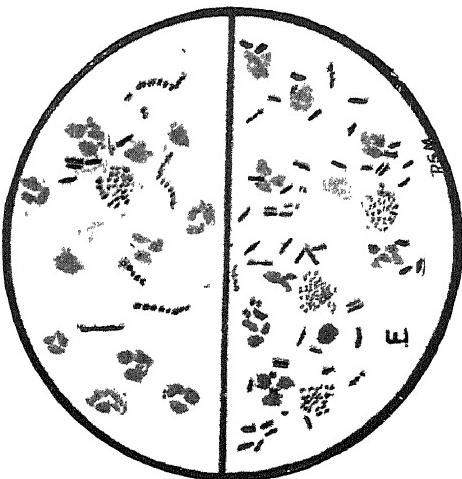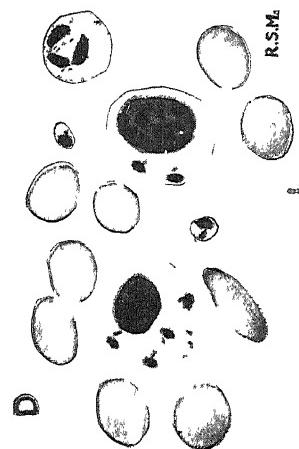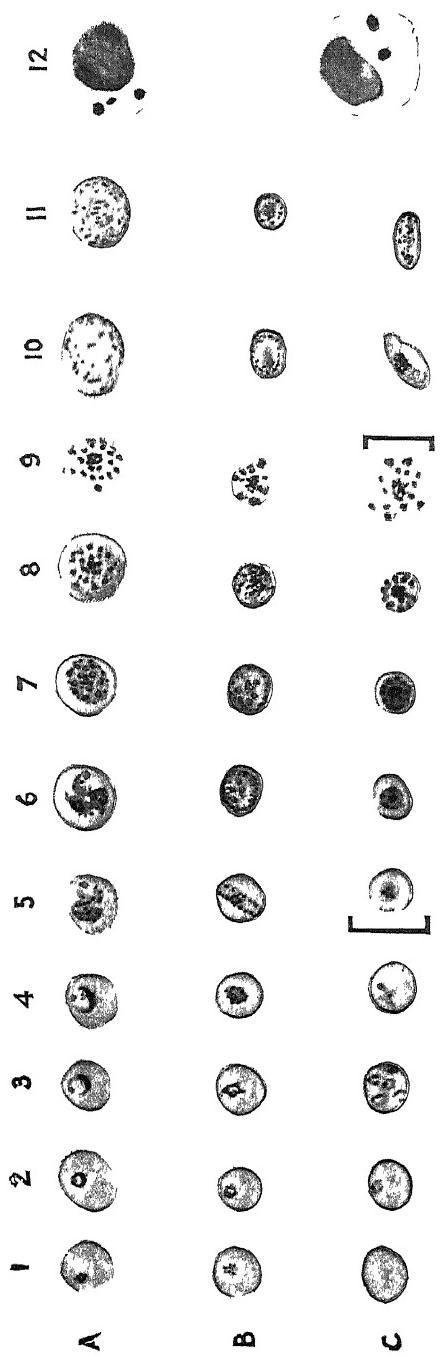
or the lid may be removed and the slides dried one to one and a half hours in an incubator.

4. Dilute 1 part of a good Giemsa stain with 6 parts of *neutral,* or only slightly alkaline, distilled water (P^H 7.0 to 7.2) just before use. Cover films for about one-half hour. Previous fixation with alcohol and dehemoglobinization are not required.

5. Place the slides in distilled water for about five minutes for partial decolorization. The time required depends upon the dilution of stain, the amount used and the thickness of the smears. If the background is deep blue and the leukocytes almost black the preparation is overstained.

6. Drain and allow to dry at room temperature or in an incubator. Examine with oil-immersion lens.

7. Except in the case of crescents, it is unsafe to call anything a parasite unless it shows a red chromatin dot, or mass, associated with blue cytoplasm. Bacteria may prove deceptive; likewise dirt. Do not reckon anything as a parasite which can be interpreted as an artefact. It is well to examine the margin of the film where the erythrocytes are partially fixed by drying.

8. In most specimens the *benign tertian parasites* appear in different stages of growth, and it will be found that older schizonts (plasmodia) are easily recognized by their larger size, irregular form and abundant chromatin and pigment. In some thick films the outline of the enlarged host cell persists, even at the center of the preparation; at the margin of the preparation the host cell often remains intact and may exhibit Schuffner's dots. Benign tertian rings or younger parasites usually have larger chromatin dots and more abundant cytoplasm than do estivo-autumnal rings. When the cytoplasm occurs in ring form, its outline is less regular than in estivo-autumnal.

9. *Quartan (Plasmodium malariae)* is regarded by some authors as hardly distinguishable from benign tertian in thick films. In most cases, however, the smaller and more compact rings and schizonts, and the more abundant pigment, serve to distinguish them. In sporulating forms the quartan has only 8 spores while the benign tertian has 16 or more.

10. In *estivo-autumnal (Plasmodium falciparum)* the rings may vary greatly in size, but are generally smaller than those of benign tertian. Chromatin dots are smaller and more often two dots occur in one ring. Where many small rings and no plasmodia are seen, it is usually safe to identify the parasite as estivo-autumnal. Crescents, characteristic of estivo-autumnal, are easily recognized when typical in form; but they sometimes change their shape in slowly drying films and may assume more rounded forms, which may simulate the schizonts of benign tertian or quartan. The more compact pigment of crescents, the deeper staining of the cytoplasm, and, in some cases, a pink remnant of the host cell will aid in identifying doubtful forms. At the margin of thick films, crescents dry more rapidly and are more likely to retain their typical form. Except crescents, larger parasites of estivo-autumnal are very rare in the peripheral blood.

Concentration Method of Bass and Johns.—1. Draw 10 c.c. of blood from a vein and place in a tube carrying 0.2 c.c. of the following:

Sodium citrate	5 gm.
Dextrose	5 gm.
Water (distilled)	10 c.c.

Dissolve with aid of heat.

2. Divide the blood between two centrifuge tubes and centrifuge at high speed (2500 revolutions per minute) for the proper length of time (about five minutes for a centrifuge with a radius of 18 centimeters).

3. With a capillary pipet remove the supernatant plasma. Then carefully skim off the grayish layer of leukocytes and parasites and place in a tube about 12 centimeters long with an inside diameter of about 0.5 centimeter (made from ordinary glass tubing). Add an equal volume of plasma.

4. Mix and centrifuge as before.

5. With a capillary pipet draw off the "cream." Mix by forcing in and out upon a slide. Then draw into the pipet and seal the tip in a flame. Nick with a file and break off above the blood column.

6. Place this slender tube in the centrifuge and centrifugate again as above.

7. The leukocytes will form a grayish layer upon the surface of the sediment. This and the upper portion of the erythrocyte layer contain the parasites.

8. Nick with a file and break off the capillary tube at a point 1 to 2 millimeters below the bottom of the leukocyte layer.

9. With a capillary pipet, the stem of which will pass inside the capillary tube, remove the small amount of red cells and leukocytes together with a little plasma.

10. Mix well, make smears on slides, and stain with Wright's stain in the usual way.

11. Best results are obtained with estivo-autumnal crescents and adult tertian and quartan parasites. Very young parasites do not concentrate as well, if at all.

EXAMINATION OF BLOOD FOR FILARIA

Wet Method.—1. Puncture finger or lobe of ear and place large drop of blood on slide.

2. Immediately cover with cover glass and examine with low-power lens.

3. The larvae can be located by the disturbance they produce among the corpuscles.

The larvae (*Filaria bancrofti*) are slender, about as wide as a red cell and about 0.2 to 0.4 millimeter long (Fig. 69). Some species appear periodically in the peripheral circulation and, in the case of *Filaria bancrofti,* they are most numerous at night. It is best to examine for this parasite at about 2 A.M. Should the above examination fail to reveal larvae, the following method is recommended:

Concentration Method.—1. Collect 1 c.c. of blood from ear or finger puncture in 5 c.c. of 2 per cent acetic acid.

2. Mix well and centrifuge.

3. Spread sediment on slide, cover with cover glass and examine with low-power lens.
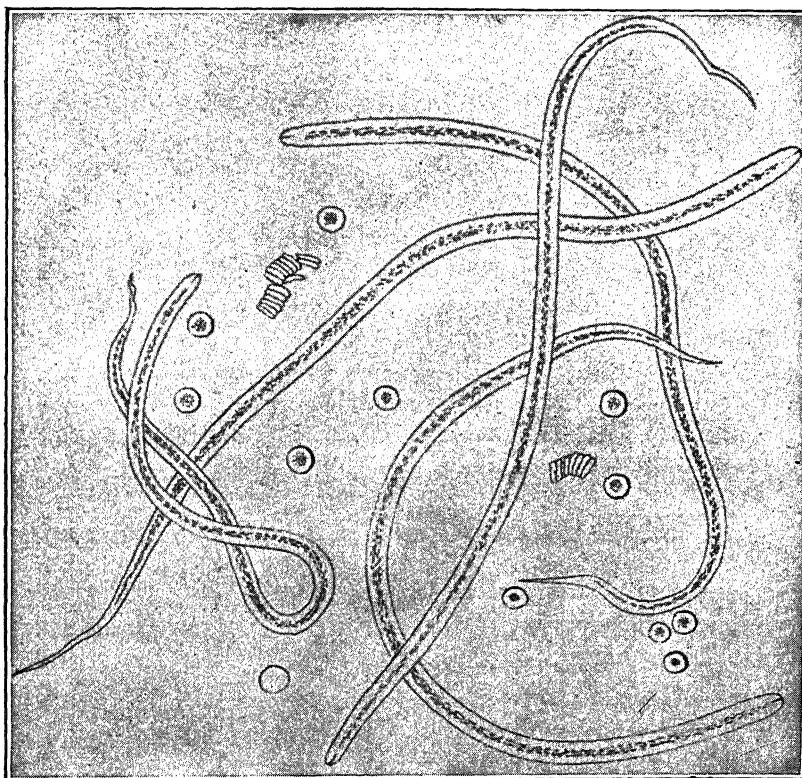
FIG. 69.—LARVAE OF FILARIA BANCROFTI.

(After Railliet. From Braun, *Die thierischen Parasiten des Menschen*, Bale Sons and Danielsson, London.)

Staining Method.—1. Make blood smears in usual manner or from sediment obtained by the above concentration method.

2. Dry, fix and stain by one of the methods for staining blood smears.

DETECTION OF TRICHINELLA SPIRALIS

Examination of Blood for Larvae.—1. The larvae are sometimes found in the blood between the sixth and twenty-second days after the onset of symptoms.

2. A high eosinophilia is usually seen in differential leukocyte counts.

3. Obtain 10 c.c. of blood by venous puncture.

4. Mix thoroughly with 25 c.c. of 2 per cent acetic acid.

5. Centrifuge thoroughly.

6. Examine the sediment either moist by placing a drop on a slide and covering with cover glass or by smears stained with Wright's stain.

7. The larvae are easily recognized.

8. The results will be positive in about 50 per cent of the cases.

9. The larvae are also sometimes encountered in the spinal fluid after thorough centrifuging and preparing smears of sediment.

Examination of Muscle.—1. Tease out bits of muscle (removed from the pectoralis major, deltoid, biceps or gastrocnemius muscles) on slides in drops of decinormal sodium hydroxide solution.

2. Cover with cover glasses and examine with 1/6 or oil-immersion lens.

3. The coiled larvae are easily seen (see Fig. 139).

DIAGNOSIS OF TRYPANOSOMIASIS

Several important diseases of man and the lower animals are caused by species of the genus Trypanosoma:

Disease	Host	Trypanosome
African sleeping sickness..	Man	T. gambiense
Chaga's disease..................	Man	T. cruzi
Surra.........................	Horse	T. evansi
Nagana.......................	Horse, cattle, sheep, goat and dog	T. brucei
Dourine.................... ..	Horse	T. equiperdum

Stained Blood Smears.—1. Prepare blood smears same as for differential blood count.

2. Stain with Wright's stain.

3. The organisms are never found within the cells but are always free in the blood plasma (Fig. 70).

4. In the early stage of sleeping sickness and during the febrile stage of Chaga's disease, the organisms can usually be found by this method.

Examination of Lymph Glands.—1. Aspirate a lymph gland with a large hypodermic needle and syringe. It is sometimes necessary to first inject a few drops of sterile saline solution.

2. Prepare smears and stain with Wright's stain.

3. This method is recommended when the peripheral blood examination is negative for *Trypanosoma gambiense.*

Examination of Spinal Fluid.—1. Make spinal puncture and withdraw about 10 c.c. of fluid.

2. Centrifuge for fifteen minutes.

3. Prepare smears of the sediment and stain with Wright's stain.

4. The spinal fluid examination is useful in the late stages of sleeping sickness when the other methods are negative.

Concentration of Blood.—1. Thoroughly centrifuge 10 c.c. of citrated blood.

2. Make smears from the leukocytic cream layer, which is just above the packed red cells.

3. Stain with Wright's stain.

Animal Inoculation.—1. Inoculate white rats intraperitoneally with 1 c.c. of blood or tissue juice from suspected case. Rats and mice are particularly susceptible. Guinea-pigs and rabbits are also, but to a less extent. For *Trypanosoma gambiense* monkeys are preferred.

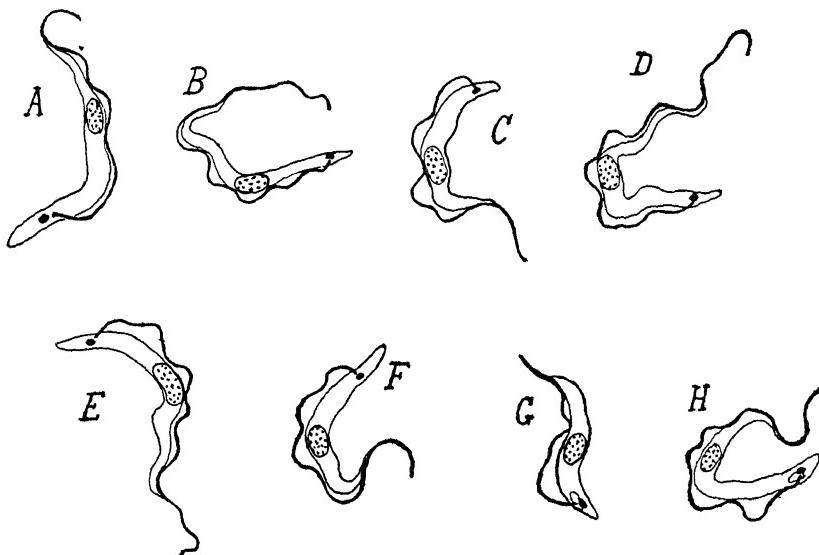2. Make daily blood smears. In positive cases the trypanosomes will

Fig. 70.—The Most Important Trypanosomes Parasitic in Vertebrates.
A. Tr. lewisi. B. Tr. evansi (India). *C. Tr. evansi* (Mauritius). *D. Tr. brucei. E. Tr. equiperdum. F. Tr. equinum. G. Tr. dimophon. H. Tr. gambiensi.* × 1500. (After MacNeal.)

appear between the third and fourteenth day and remain in the blood stream quite constantly.

Complement-Fixation Test.—This test has been found useful in the diagnosis of dourine and the detection of carriers among the lower animals. Horses may tolerate an infection for one to three years, during which time they are capable of conveying the disease and yet remain normal in health and general appearance, and this method of diagnosis is, therefore, invaluable (Tyzzer). The test is similar to any other complement-fixation test except for the preparation of the antigen, a description of which is given in the section on serology. The test gives a group reaction, so the same antigen can be used for all species.

DETERMINING THE COAGULATION TIME OF THE BLOOD

Principles.—1. When the coagulation time of the blood is determined by methods using finger puncture to procure the blood, the results are only approximately correct and not entirely dependable, because of the admixture of tissue juices with the blood.

2. The coagulation time is prolonged in hemophilia, melena neonatorum, obstructive jaundice, some anemias and leukemias and some of the infectious diseases.

Capillary Tube Method.—1. Cleanse a finger and puncture as for blood count.

2. Fill a capillary glass tube (1.5 millimeters in diameter and 3 to 5 centimeters long) with blood. The tube will fill readily by capillary attraction if one end touches the drop of blood and the tube is inclined downward. *Note the time.*

3. At half-minute intervals after an interval of three minutes carefully break a small piece off the end of the tube, holding it in such a manner that the broken ends are kept together; then separate the ends slowly and note if fibrin threads span between the ends. When the threads are seen to spread a distance of 5 millimeters or more, *note the time.*

4. *The time between the filling of the tube and the appearance of fibrin threads is the coagulation time.* The normal is from two to eight minutes.

Drop Method.—1. Cleanse and puncture finger as for blood count (puncture deep to insure free flow of blood).

2. Place several drops on a clean slide (the drops should be about 4 or 5 millimeters in diameter). *Note the time.*

3. At half-minute intervals draw a needle through one of the drops As soon as the needle picks up fibrin threads and drags them along, coagulation has taken place. *Note the time.*

4. The time interval between placing the drop on the slide and the formation of fibrin shreds is the *coagulation time. The normal time is between two and eight minutes.*

Venous Puncture Method (Lee and White).—1. With a small syringe, fitted with a gauge 20 needle, puncture a vein at the elbow and collect 1 c.c. of blood without using suction. *Note the time.*

2. Remove the needle from the syringe and place the blood in a test tube having a diameter of 8 millimeters. The test tube should be absolutely clean and rinsed with physiological salt solution just before the blood is placed in it.

3. Set the tube upright in a rack at room temperature or better in a water bath or glass of water at a temperature of 75° F.

4. At one-minute intervals tilt the tube to see if the blood still flows. As soon as it fails to flow and can be inverted, coagulation has taken place.

5. The interval between the time the blood is removed from the vein and

the time the tube can be inverted without disturbing the clot is the *coagulation time*. The normal time is from five to ten minutes.

6. A control test is advised with the blood of a normal person.

Modification of the Brodie-Russell-Bogg Method. —1. The finger or ear is washed with alcohol and allowed to dry; puncture and wipe away first drop of blood.

2. Note the time on watch when second drop appears; place drop on cone of coagulator and adjust in moist chamber (Figs. 71 and 72).

3. Place chamber on microscope stage and examine with low-power objective.

4. Squeeze rubber bulb (not too forcibly) about once every half to one minute, noting effect of air on corpuscles.

5. When the movement of individual corpuscles ceases and the drop moves as a whole or the corpuscles move toward the center and spring back to the periphery, note the time. The interval represents the coagulation time.

6. Duplicate readings should be made after the cone has been made perfectly clean and dry.

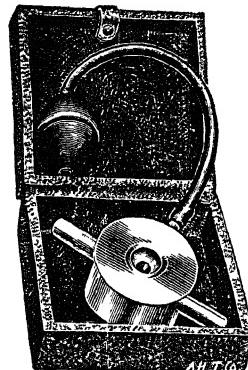7. Normally the blood coagulates in two to eight minutes.

FIG 71. — BRODIE-RUS-SELL - BOGGS COAGU-LOMETER

FIG. 72.—CROSS SEC-TION OF COAGULOM-ETER.

DETERMINING THE BLEEDING TIME OF BLOOD

Principles.—1. This test of the blood is useful in the diagnosis of hemorrhagic diseases and with the coagulation time as a preoperative test.

2. The normal bleeding time is from one to three minutes. It is prolonged in cases where the blood platelets are markedly reduced and in chloroform and phosphorus poisoning; also in liver diseases which show a tendency to hemorrhage. In hemophilia the bleeding time is usually normal.

3. It is dependent upon the mechanical and probably the chemical action of the blood platelets; also upon the elasticity of the skin and the ability of the tissue juices to promote clotting.

Procedure.—1. Puncture the lobe of the ear or the finger so that the blood flows drop by drop without any assistance.

2. Note the time the first drop appears.

3. Remove with filter paper each drop as it forms, care being taken not to touch the skin.

4. Note the time bleeding stops.

5. The time interval between the appearance of the first drop and the removal of the last represents the bleeding time. Normally it is two to three minutes.

DETERMINING THE PROTHROMBIN TIME OF BLOOD

This test is useful in the diagnosis of hemophilia. The prolonged *prothrombin time* in this condition is probably due to the failure of the platelets to disintegrate and release thromboplastic material.

Howell's Method.—1. Secure 2 c.c. of blood by venous puncture in a syringe which has just been washed with physiological salt solution and without using suction.

2. Immediately place in a test tube containing 0.25 c.c. of a 1 per cent solution of oxalate in physiological saline solution.

3. Mix thoroughly by inverting and centrifuge.

4. Remove the clear plasma and place 5 drops in each of four small test tubes.

5. Add 0.5 per cent calcium chloride as follows:

Tube 1.................................... 2 drops
Tube 2.................................... 3 drops
Tube 3.................................... . 4 drops
Tube 4.................................... .... 5 drops

6. Note the time.

7. Mix gently and observe coagulation in the same manner as described above under venous puncture method for coagulation time. When a tube can be inverted without disturbing the clot, coagulation is complete. Note the coagulation time of the tube which coagulates first. This is the *prothrombin time*. Blood from a normal person should be tested at the same time as a control and for determining the *prothrombin quotient* of Jurwitz and Lucas:

$$\frac{\text{prothrombin time of unknown}}{\text{prothrombin time of normal}} = \text{prothrombin quotient}$$

8. The normal prothrombin time is about ten minutes.

9. The normal prothrombin quotient is about 1 and in hemophilia from 5 to 25.

DETERMINING THE CALCIUM TIME OF BLOOD

This test is used for determining if in cases of prolonged coagulation time the delay in coagulation is due to a deficiency of calcium.

Procedure.—1. Secure 2 or 3 c.c. of blood by venous puncture.

2. Place 1 c.c. of blood in each of two test tubes having a diameter of 8 to 10 millimeters.

3. To one of the tubes add 3 drops of a 1 per cent solution of calcium chloride.

4. Observe coagulation. If the tube containing the calcium coagulates within the normal time and the tube without calcium shows delayed coagulation, the prolonged coagulation time of the blood is considered as being due to a deficiency in calcium.

DETERMINING THE CLOT RETRACTION TIME

After coagulation has taken place the clot will contract and express serum. This is called retraction and the phenomenon appears to have some relation to the platelets. If the platelets are present in normal number, retraction occurs; if the platelets are greatly diminished, retraction will be retarded or absent. The test has no relation to the coagulation time even in hemophilia where the retraction is normal.

Procedure.—1. Secure 2 or 3 c.c. of blood by venous puncture.

2. Place in test tube and incubate at 37° C.; observe occasionally for a period of a day or two.

3. The first evidence of retraction is the separation of the clot from the wall of the tube and then the gradual expression of serum. Normally retraction is completed in from eighteen to twenty-four hours. In purpura it may be retarded or absent.

DETERMINING THE TONICITY OR FRAGILITY OF ERYTHROCYTES

Principles.—1. Normally human erythrocytes carefully collected against injury can remain for two hours at room temperature in solutions containing 0.42 to 0.44 per cent sodium chloride *before hemolysis begins,* while under these conditions *hemolysis is complete* in 0.36 to 0.32 per cent solutions.

2. The point of beginning hemolysis is called *minimal resistance,* while the point of complete hemolysis is called *maximal resistance.* The former is of more diagnostic value than the latter.

3. After splenectomy resistance increases; in various diseases of the blood, variations from the normal may occur.

Sanford's Method.—1. Place twelve small test tubes in a rack and mark them from 25 to 14.

2. In each tube place as many drops of accurately prepared 0.5 per cent salt solution as the number indicated on the tube. To insure equality in size of drops the pipet must be always held at the same angle. The 0.5 per cent salt solution is prepared by placing C.P. sodium chloride in an oven for thorough drying and then dissolving 0.5 gram in exactly 100 c.c. of distilled water.

3. With the same pipet add sufficient number of drops of distilled water to each tube to make the total volume 25 drops.

4. Take the tubes in a rack to the bedside of the patient. Obtain 1 or 1.5 c.c. of blood from a vein with a small dry syringe and No. 21 needle and at once add 1 drop to each tube (Fig. 73).

5. If some time must elapse before the blood can be added, it may be mixed with 5 volumes of a 1 per cent solution of sodium citrate in physiological saline solution. Mix well. Centrifuge. Discard the supernatant fluid. Add an equal volume of saline solution to the corpuscles (gives a 50 per cent suspension) and add 1 drop to each tube.

6. It is advisable to prepare a similar set of tubes, using the blood of a normal person as a control.

7. Allow the tubes to stand at room temperature for two hours.

8. Read the results. Reading should be made from tube marked 25 to tube marked 14, noting number on tube in which hemolysis begins and the first tube showing complete hemolysis.

9. Multiply the number on the tube by 0.02 to obtain the percentage of sodium chloride it contains.

10. Normal blood *begins* to hemolyze in 0.42 or 0.44 per cent sodium chloride and is *completely* hemolyzed in 0.36 to 0.32 per cent. When a control is used, a variation of 0 02 or 0.04 may be considered quite definite. Sanford found the average figures for beginning and complete hemolysis in 23 cases
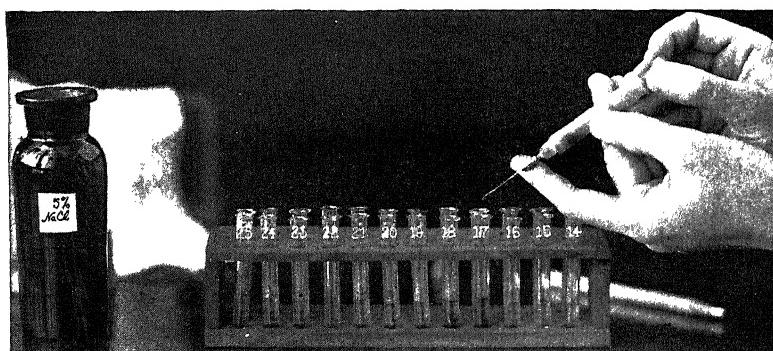

FIG 73 —FRAGILITY TEST; ADDING THE BLOOD.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co)

of hemolytic jaundice to be 0.478 and 0.413 respectively; in chronic obstructive jaundice, 0.396 and 0.31. In secondary and pernicious anemia the figures vary only slightly from the normal, with a tendency to slight increase of resistance. In purpura, resistance is normal.

DETERMINING THE SEDIMENTATION TIME OF ERYTHROCYTES

Principles.—1. It has been known for centuries that in some acute infectious diseases the erythrocytes may become separated from the plasma even before coagulation has occurred. During recent years a number of methods have been devised for determining the sedimentation time of these cells, especially in pregnancy, tuberculosis, and acute bacterial infections.

2. An increase or change in the electrical charge of the erythrocytes, an increased cholesterol content, changes in viscosity of the plasma, variations in the erythrocyte count and certain chemical changes in the blood have been ascribed as causative factors, but none have been definitely proven or accepted

although it appears that the number of erythrocytes greatly influences the speed of sedimentation.

Cutler Vein Method.—1. Cleanliness and dryness of apparatus are essential. If any clotting occurs, the test must be done over.

2. Apply tourniquet to arm.

3. Aspirate into syringe 0.5 c.c. of 3 per cent sodium citrate solution.

4. Puncture cubital vein and draw blood to 5 c.c. mark.

5. Release tourniquet and withdraw needle from vein.

6. Draw back the barrel of the syringe about 1 centimeter and gently tilt the syringe backward and forward several times to insure uniform mixing of blood and citrate solution.

7. Remove needle from syringe, as it may contain clotted blood, and pour contents into the sedimentation tube (Fig. 74).

8. When it is necessary to obtain specimens from several patients, let this tube stand in the rack without paying any attention to it.

9. Wash syringe in water, wipe barrel with a clean cloth and then pass syringe through alcohol and ether and dry in air; this insures a clean and dry syringe, which is again ready for use.

10. After all the samples of blood have been taken they are carried to the laboratory; it is best to number each tube to avoid possible error, and to carry the tubes in a rack.

11. Before any readings are made, each tube is stoppered with a paraffin-coated cork and gently turned upside down two or three times; this insures a uniform distribution of the red blood cells, for by the time the tubes are brought to the laboratory, in many cases marked sedimentation has already taken place.

FIG. 74.— CUTLER BLOOD SEDIMENTATION TUBE.

12. The tubes may be allowed to stand as long as ten hours before making any readings without losing valuable information or noticing any practical change in the character of the graph. After ten hours the sedimentation phenomenon begins to disappear, especially in those cases where there has been a marked and rapid sedimentation.

13. The position of the sedimenting column of red blood cells is read every five minutes for one hour. This is done with ease as the boundary zone between the red blood cells and the plasma is usually sharp and distinct. The observations are recorded on the sedimentation charts on which the horizontal lines represent the divisions on the sedimentation tube, and the vertical lines the intervals of time. In this way a graph is traced which shows the position of the sedimenting column of red blood cells at any period of time during the first hour.

14. The sedimentation value is determined according to the path traversed by the red blood cells during the first hour and depends upon the nature of the graph, the sedimentation index, and the sedimentation time. Together they furnish all the information that is likely to be obtained from the sedi-

mentation test. The graph (Fig. 75) serves as a rough estimation of the presence or absence of pathologic activity. The sedimentation index and sedimentation time help to determine the degree.

15. As can be seen from Figure 75, there are four distinct graphs, two of which are straight lines and two of which are curves. From the nature of the graph, they are called horizontal line, diagonal line, diagonal curve, and vertical curve. A horizontal line within the meaning of the sedimentation reaction may be defined as a straight line, with a sedimentation index falling within normal limits, and a sedimentation time always longer than sixty minutes.

16. By *sedimentation index* is meant the total sedimentation of the red blood cells at the end of sixty minutes expressed in millimeters. The normal sedimentation index for men varies from 2 to 8 millimeters, with an average of 3 to 4; for healthy women from 2 to 10 millimeters, with an average of 5 to 6; during menstruation probably as high as 12 millimeters because the erythrocytes become packed in the bottom of the tube as closely as possible.

17. By *sedimentation time* is meant the number of minutes that elapse before the period of packing of the red blood cells sets in. The normal sedimentation time is always a question of hours. A diagonal line is a straight line, with a sedimentation index falling outside of normal limits and a sedimentation time of sixty minutes or more. A diagonal curve is a curve of gradual slope, with a sedimentation index falling outside of normal limits and a sedimentation time of sixty minutes or less. A vertical curve is a curve of sharp slope, with a sedimentation index falling outside of normal limits and a sedimentation time always less than sixty minutes.

18. It is not always a special or specific test for any disease and is not in any sense diagnostic, as the rate of sedimentation is accelerated not only in active tuberculosis but also in acute inflammations of other origin as well as in carcinoma and the toxemias.

It is of most value in distinguishing between noninflammatory and inflammatory processes and for estimating the activity or progress of pulmonary tuberculosis. A normal curve is seldom seen in the active stages of this disease. In the persistent absence of increased rate of sedimentation over a period of about ten days, one can state with considerable certainty that there is no active tuberculous process in existence. It also serves to indicate the degree or severity of other inflammatory states, the rate of sedimentation being greater in the acute states. In cancer an actively growing and fungating tumor is apt to give a greater rate than a slowly growing scirrhous carcinoma.

Cutler's Finger-Puncture Method.—1. Select one of the fingers of the hand and carefully cleanse the distal phalanx with an alcohol sponge, rubbing briskly to induce hyperemia. It is sometimes of service to immerse the finger in hot water for a few minutes, especially in anemic individuals or where the skin is thicker than usual.

BLOOD SEDIMENTATION TEST

Case No._____   Date_____19____

Name_____   Tube No._____

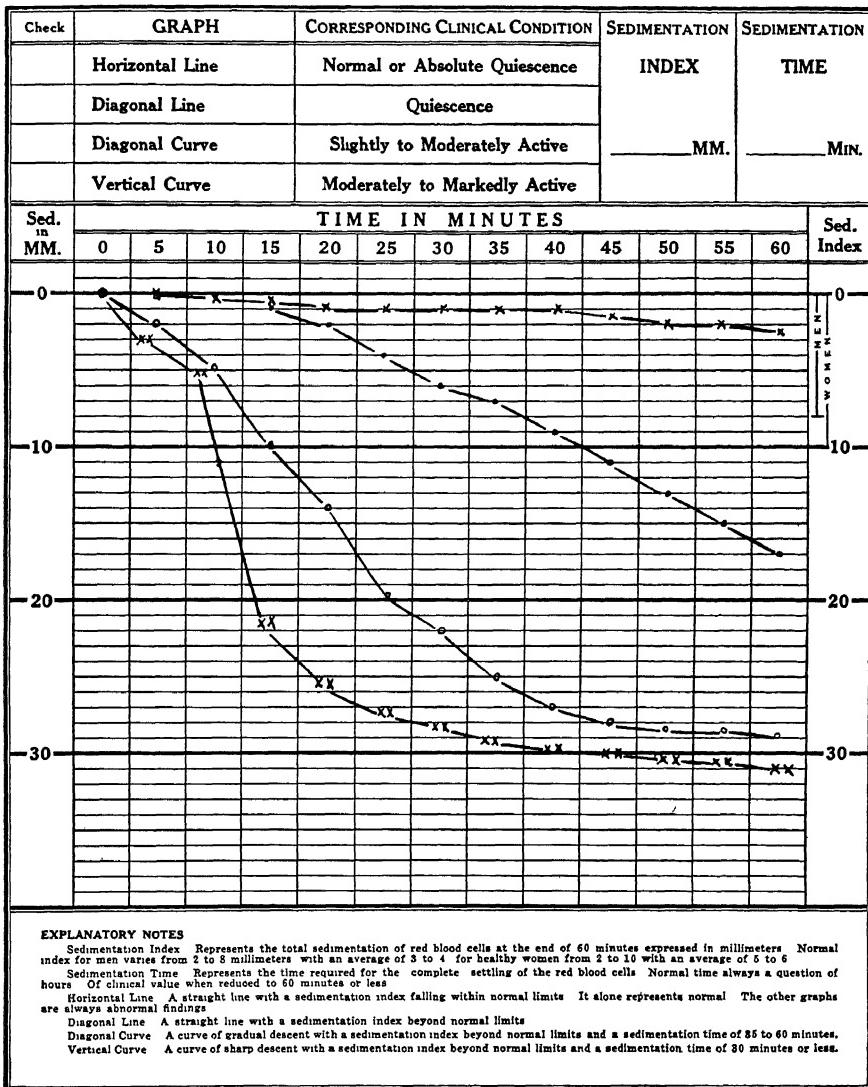Address_____   Diagnosis_____

Check	GRAPH	CORRESPONDING CLINICAL CONDITION	SEDIMENTATION INDEX	SEDIMENTATION TIME
	Horizontal Line	Normal or Absolute Quiescence		
	Diagonal Line	Quiescence		
	Diagonal Curve	Slightly to Moderately Active	____MM.	____MIN.
	Vertical Curve	Moderately to Markedly Active		

EXPLANATORY NOTES

Sedimentation Index   Represents the total sedimentation of red blood cells at the end of 60 minutes expressed in millimeters   Normal index for men varies from 2 to 8 millimeters with an average of 3 to 4 for healthy women from 2 to 10 with an average of 5 to 6

Sedimentation Time   Represents the time required for the complete settling of the red blood cells   Normal time always a question of hours   Of clinical value when reduced to 60 minutes or less

Horizontal Line   A straight line with a sedimentation index falling within normal limits   It alone represents normal   The other graphs are always abnormal findings

Diagonal Line   A straight line with a sedimentation index beyond normal limits

Diagonal Curve   A curve of gradual descent with a sedimentation index beyond normal limits and a sedimentation time of 35 to 60 minutes.

Vertical Curve   A curve of sharp descent with a sedimentation index beyond normal limits and a sedimentation time of 30 minutes or less.

FIG 75—BLOOD SEDIMENTATION CHART AND GRAPHS.

Horizontal line represents clinically healthy individual; diagonal line represents clinically quiescent tuberculosis; diagonal curve represents clinically slightly active tuberculosis; vertical curve represents clinically marked active tuberculosis.   (J. W. Cutler.)

2. Puncture distal phalanx on its palmar surface and toward either side. This insures an easier flow of blood.

3. Collect blood in storage tube until about half filled (0.5 c.c.) (Fig. 76). The storage tube, which must be clean and dry, is first filled with 3 per cent sodium citrate solution and its contents emptied. The quantity of citrate solution clinging to the walls of the tube is sufficient to prevent clotting of blood. From time to time the finger tip and the rim of the storage tube should be wiped with the citrate solution to remove possible clotted particles, and the tube shaken as a further safeguard to prevent clotting. This is important. If clotting occurs the test must be repeated. In general the less the time consumed in obtaining the quantity of blood desired and the greater the ease with which the blood is obtained, the less the likelihood of the blood clotting and also the more accurate the result.

FIG 76 — CUTLER BLOOD COLLECTING TUBE.

4. If samples of blood are to be obtained from several patients, place the storage tube in its rack and pay no more attention to it until all the samples have been obtained. Cork each storage tube with a paraffin-coated cork to safeguard against accident.

5. When ready to make readings (several hours may elapse with safety, after taking the samples of blood), shake each storage tube gently but thoroughly to insure uniform distribution of blood cells, for in many instances marked sedimentation may occur by the time one is ready to make readings.

6. Draw blood into the sedimentation pipet (Fig. 77) up to the zero graduation and attach spring cap to bottom of pipet. Draw the blood into the pipet by means of mouth suction on the rubber tube until the stem is filled with a *solid* column of blood, then holding the pipet in a horizontal position, remove the rubber tube and with pressure of the finger tip on the opening of the pipet, reduce the column of blood until the top is exactly at the zero mark.
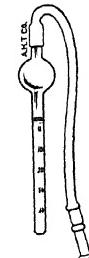
FIG. 77. — BLOOD SEDIMENTATION PIPET.

7. Place the pipet in the sedimentation rack and determine the change in velocity by observing the position of the upper level of the sedimenting column of red blood cells every five minutes for one hour. These observations are recorded on the sedimentation charts, on which the horizontal lines represent the divisions on the stem of the pipet and the vertical lines the intervals of time. In this way a graph is traced which shows the position of the sedimenting column of red blood cells at any period of time during the first hour.

CHAPTER VI

METHODS FOR THE EXAMINATION OF URINE

COLLECTION AND PRESERVATION

Principles.—1. As the excretion of sugar, albumin, casts, pus and other important urinary constituents is known to vary at different times, the examination of specimens collected over twenty-four hours is recommended, *especially for quantitative tests.* The next best is a mixture of night and morning urine.

2. Single specimens continue to be mostly employed because of greater convenience in collection but should be used mainly for routine qualitative tests.

3. Single specimens collected at varying times in the day may yield different results, especially in amounts of sugar and albumin; this is a frequent reason for varying reports from different laboratories examining specimens of urine of the same person collected at different times. *Single specimens should be labeled with the time of voiding.* Specimens voided two or three hours after a meal are likely to contain most sugar or albumin; those passed first in the morning are least likely to contain them.

4. Different methods and variations in technic and skill, however, probably account for most of the discrepancies in reports from different laboratories.

5. Containers used for collection of urine should be *chemically clean* and preferably sterile. Careful cleaning is especially required in hospital laboratories to avoid the possibility of carrying over traces of albumin and sugar. Traces of syrup in insufficiently washed medicine bottles are sometimes responsible for mistakes.

6. Contamination with vaginal discharges may account for the presence of albumin and pus; contamination with menstrual discharges may account for the presence of albumin and blood. Both should be carefully avoided, as well as contamination with feces.

7. From 3 to 8 ounces of urine should be submitted for ordinary examination.

8. A preservative is advisable if examination is delayed for twenty-four hours or longer as in the case of urine sent by mail and in the collection of twenty-four-hour specimens.

9. Urine to be examined for tubercle bacilli may be voided, although there are chances of contamination with smegma bacilli (see Chapter XIX).

Urine for other bacteriological examinations should be collected aseptically by sterile catheter into sterile containers (*without a preservative*), as it is almost impossible otherwise to avoid bacterial contamination, especially with *B. coli* and staphylococci.

FIG. 78—URINE TUBE. (Pons and Krumbhaar)

Methods.—1. Give specific directions for collecting twenty-four-hour specimens. A large bottle or other container kept in a cool place may be employed. The total amount should be *carefully mixed and measured* and 6 to 8 ounces submitted for examination. A preservative may be required.

2. In hospital laboratories the urine tubes (Fig. 78) and transportation rack (Fig. 79) devised by Pons and Krumbhaar are recommended. The former are 7½ inches long and 1¾ inches wide with an approximate capacity of 90 to 100 c.c. They are conical for the settling of sediments and wide enough for taking the specific gravity of specimens. Each should be numbered.

3. Special methods must be employed for the collection of urine from infants.

Preservatives.—Decomposition sets in rapidly, especially in warm weather, and greatly interferes with all examinations. An ideal preservative should prevent the growth of bacteria and molds; should not interfere with the accuracy of physical, chemical and microscopical examinations; should be readily soluble, of low cost and preferably a solid. For *quantitative* tests the total twenty-four-hour urine is advisable and, unless kept at a low temperature, a preservative is generally required:

1. *Thymol,* if used, should not exceed 0.1 gram per 100 c.c. of urine. An excess may interfere with albumin determinations. It is not as good as formerly surmised and is unsatisfactory when urine contains sugar, acetone or diacetic acid; also when urine is to be examined for phenol and quantitatively for phosphates or magnesium.

FIG. 79—TRANSPORTATION RACK. (Pons and Krumbhaar)

2. *Formalin in proportion of 2 to 4 drops to the ounce is the most satisfactory of all, especially for the preservation of the formed elements.* An excess may interfere with tests for indican, albumin and sugar, and produce a precipitate.

3. *Boric acid,* 5 grains for each 4 ounces, delays decomposition but may interfere with sugar determination and precipitate rhombic crystals of uric acid.

4. *Toluol* may be used, especially for specimens to be examined for acetone and diacetic acid. Simply overlay the urine.

5. Chloroform is the least satisfactory and should not be used as it interferes with sugar determination and microscopical examinations.

PHYSICAL EXAMINATION

Color.—1. The color of urine is subject to wide variations but possesses some diagnostic importance.

2. Normally it is yellow or reddish-yellow (amber) due to the presence of several pigments, chiefly urochrome.

3. Color depends largely upon the concentration of urine. Dilute urines are usually pale while concentrated urines are dark. Acid urine is usually darker than is alkaline urine.

4. Color may be greatly changed by abnormal pigments and by various drugs and poisons as follows:

> Blood: red or brown; smoky
> Bile: yellowish or brown, turning greenish; yellow foam
> Chyle: milky
> Methylene blue: greenish-blue
> Phenols: olive-green to brownish-black, etc.

5. For uniformity in recording color, Vogel's scale is recommended, the urine being filtered and viewed by transmitted light in a glass 3 or 4 inches in diameter: pale yellow, light yellow, yellow, reddish-yellow, yellowish-red, red, brownish-red, reddish-brown and brownish-black. To these may be added greenish-yellow, olive, milky, etc.

Transparency and Sediments.—1. Freshly passed urine is usually clear or transparent, but may be cloudy due to the presence of phosphates or pus. The former disappears upon the addition of acid; the latter does not, but may become gelatinous (Donné's test). A freshly passed urine may also be cloudy with bacteria or comparatively clear with numerous shreds of mucopurulent material (chronic urethritis).

2. A record of the transparency is only of value in comparatively fresh specimens. All become cloudy with bacteria and alkaline salts upon standing as the result of decomposition.

3. Upon cooling and standing all specimens develop a faint cloud of mucus, leukocytes and epithelial cells which settle to the bottom—the so-called "nubecula." This has no significance.

4. Acid urines may develop a white or pinkish sediment of amorphous urates.

5. Alkaline urines may develop a heavy white sediment of amorphous phosphates.

6. Pus gives a heavy mucoid whitish sediment.

7. Blood gives a reddish-brown smoky sediment.

8. Bacteria give a uniform cloudiness which cannot be removed by ordinary paper filtration.

9. The following terminology is recommended:

    (*a*) Clear, slightly cloudy, very cloudy

    (*b*) Sediment: Slight, moderate or heavy; white, pinkish, red, brown, reddish-brown, etc.; shreds present or absent

DETERMINATION OF REACTION

1. Normally freshly voided urine is acid in reaction, the P^H ranging from 4.8 to 7.5 with a general average of 6. Twenty-four-hour specimens are less acid than freshly passed urine and may be neutral or even slightly alkaline as a result of standing.

2. Freshly passed urine may be neutral or alkaline as the result of the administration of alkalis, retention with "ammoniacal decomposition," etc.

3. Diet influences the reaction.

Litmus Test.—For ordinary purposes the reaction may be determined with good grades of blue and red litmus papers (Squibb's recommended):

    Blue turning red: acid
    Red turning blue: alkaline
    No change in either: neutral
    Changes both red and blue: amphoteric

Quantitative Test (Folin-Wu).—1. Use a sample of mixed twenty-four-hour urine as fresh as possible and accurately measured.

2. Place 25 c.c. in a small flask or evaporating dish. Add 2 drops of 0.5 per cent alcoholic solution of phenolphthalein and 15 grams of neutral finely pulverized potassium oxalate.

3. Shake vigorously for two minutes.

4. Immediately titrate with N/10 sodium hydroxide solution, shaking after each addition, until the first permanent pink color appears.

5. Read off amount of N/10 sodium hydroxide used.

6. Multiply by 4 to estimate amount required for 100 c.c. of urine and report accordingly (normally 25 to 40 c.c.).

7. Calculate and report amount required for total twenty-four-hour specimen. Normally 554 to 669 c.c. (may be less; depends largely on diet).

DETERMINATION OF HYDROGEN ION CONCENTRATION

True or hydrogen ion concentration acidity is preferred and may be determined with the indicator solutions of Clark and Lubs. Fresh clear urine should be used and the technic is that described in Chapter XVII for determining the P^H of culture media. The normal values lie between 4.8 and 7.5, with an average of about 6.0 (for vegetarians about 6.6).

DETERMINATION OF SPECIFIC GRAVITY

1. The normal average is from 1.015 to 1 020. Pathologically it may vary from 1.001 to 1.060. If the specimen contains but a small or average amount of sediment it makes but little or no difference whether the urine is mixed up or the specific gravity taken without mixing in order to use the sediment later for microscopical examination. If, however, there is a large amount of sediment the specific gravity is almost always increased by about 0.002 after thorough mixing.

2. For ordinary determinations the Squibb urinometer (Fig. 80) may be used but the urinometer used with the immiscible balance is probably the best on the market. It settles down quickly after spinning without bobbing or swaying, and its special scale makes it much easier to read. With the Squibb urinometer the technic is as follows:

FIG. 80 —SQUIBB
URINOMETER

FIG 81 —VOGEL
URINOMETER

(*a*) Fill the cylinder without producing bubbles. If the containers shown in Figure 78 are employed, the cylinder is not required. The specific gravity may be taken without mixing the urine.

(*b*) Float the hydrometer so that it does *not touch the bottom or sides.*

(*c*) Make the reading from the bottom of the meniscus.

(*d*) The instrument is adjusted for readings at 22.5° C. For accuracy add 0.001 to the reading for each 3° C. above this temperature and subtract 0.001 for each 3° C. below, although moderate reduction in temperature does not influence the specific gravity as much as increased temperature.

3. The Vogel urinometer (Fig. 81) is more accurate and consists of two spindles, graduated respectively from 1.000 to 1.025 and from 1.025 to 1.050.

4. For small amounts of urine, dilute with an equal volume of distilled water, mix and take specific gravity. Multiply the last two figures by 2. By this method the specific gravity is usually 0.001 to 0.002 higher. Or the Saxe urino-pyknometer (Eimer and Amend) may be used if at least 3 c.c. of urine are available.

5. The Exton immiscible balance supplied by the Emil Greiner Company may be employed for determining the specific gravity of drops of urine when only very small amounts are available, as in ureteral catheterization. The method and instrument are based on the principle of suspending the urine in an immiscible medium of the same specific gravity which is then determined

FIG 82.—EXTON'S IMMISCIBLE BALANCE

A drop of blood is shown in suspension. A special hydrometer is shown at the left.

by the usual methods, making possible rapid manipulations with minimal loss of material.

The cylindrical mixing chamber (Fig. 82) is partly filled with a mixture of varuolene (petroleum ether) and carbon tetrachloride with a specific gravity of about 1.012. The side thistle tube is filled with varuolene and the carbon tetrachloride is kept at hand in a drop bottle. A drop of the urine is immersed in the mixture, which is then easily and rapidly varied by means of the stopcock and dropper, so that the urine remains suspended near the middle. The specific gravity of the mixture is then taken by means of the Exton hydrometer, which gives the specific gravity of the urine.

ESTIMATION OF TOTAL SOLIDS

1. If the total output of urine for twenty-four hours is reported in ounces, multiply the last two figures of the specific gravity by the number of ounces voided and to the product add one-tenth of itself. This gives the total solids in grains. Example:

$$\text{Twenty-four-hour output} = 37 \text{ ounces}$$
$$\text{Specific gravity (at } 25° \text{ C)} = 1\ 014$$
$$14 \times 37 = 518 + 51\ 8 = 569\ 8 \text{ grains}$$

2. If the twenty-four-hour specimen is reported in c.c., multiply the last two figures of the specific gravity by Long's coefficient, 2 66; then multiply by the total output and divide by 1000, which gives the total solids in grams. Example:

$$\text{Twenty-four-hour output} = 1120 \text{ c.c.}$$
$$\text{Specific gravity (at } 25° \text{ C)} = 1\ 018$$
$$2.66 \times 18 = 47.8 \text{ gm in 1000 c c. of urine}$$
$$\frac{47\ 8 \times 1120}{1000} = 53\ 5 \text{ gm. in 1120 c c.}$$

3. The normal output for an adult of 150 pounds is about 60 grams or 950 grains. The above methods are only approximately correct but sufficient for clinical purposes.

4 The output of urinary solids is influenced by body weight, diet, exercise, age, metabolism and state of kidney excretion.

QUALITATIVE DETECTION OF ALBUMIN

Principles.—Normal urine contains a trace of albumin which is too slight to be detected by the simple tests in general use, a large number of which have been described. All depend upon its precipitation by chemical agents or coagulation by heat All precipitate both serum albumin and serum globulin and do not differentiate between these two proteins. Most are subject to some error largely due to the precipitation of mucin or other constituents. All require the use of clear specimens, preceded by filtration if necessary, in order to detect small amounts of albumin. The methods here given are recommended for ordinary routine work.

Methods for Filtering.—As a general rule simple filtration through ordinary filter paper is sufficient unless cloudiness is due to bacteria. Very large numbers of bacteria and especially dissolved organisms in alkaline urine may yield faint traces of albumin. They are difficult to remove but this may be accomplished sufficiently for testing by centrifuging or by adding about one teaspoonful of purified talc, infusorial earth or animal charcoal to each 2 or 3 ounces, shaking well and filtering through two thicknesses of filter paper. Some albumin is also removed by absorption.

Methods for Recording Reactions.—A wide diversity of methods for reporting qualitative tests are in use; they account in large part for discrepancies in reports from different laboratories. A uniform method and terminology are urgently needed. The following are recommended:

> — = *negative.*
>
> ± = *very slight trace.* Cloudiness or ring can just be seen against a black background.
>
> + (1) = *slight trace.* Cloud is distinct but not granular; no definite flocculation Or the ring is sufficiently definite to be seen without a black background.
>
> ++ (2) = *moderate trace.* Cloud is distinct and granular without definite flocculation. Or the ring is dense but not wholly opaque when viewed from above. Represents about 0 1 per cent of albumin.
>
> +++ (3) = *heavy cloud* Cloud is dense with marked flocculation or the ring is heavy, wholly opaque and sometimes curdy. Represents about 0 2 to 0 3 per cent.
>
> ++++ (4) = *very heavy cloud.* Heavy precipitate to boiling solid; or very dense ring. Represents 0 5 or higher per cent of albumin; 3 per cent albumin boils solid.

Fairly permanent standards, showing the clouds and precipitates but not the rings, may be prepared as follows and kept on hand for rapid comparison and more accurate readings, especially of the Purdy and heat acid tests:

1. Dissolve 20 grams of gelatin in 100 c.c. of hot water.
2. Add 0.3 c.c. of formalin. Mix well. Keep at about 50° C.
3. In six thin-walled test tubes of the kind used for the heat and acid test, place the following amounts of serum:

> No. 1: no serum   No. 4: 0.1 c.c. undiluted
>
> No. 2: 0.2 c.c. of 1:10   No. 5: 0.3 c.c. undiluted
>
> No. 3: 0.5 c.c. of 1:10   No. 6: 0.6 c.c. undiluted

4. To each tube add enough water to make 2 c.c.
5. To each tube add 8 c.c. of hot formalized gelatin.
6. Mix well by inverting several times.
7. Heat the upper third of each tube in a Bunsen to coagulate the albumin.
8. Cork and label each as follows:

> No. 1: negative —
>
> No. 2: very slight trace (±)
>
> No. 3: slight trace (+)
>
> No. 4: moderate trace (++)
>
> No 5: heavy cloud (+++)
>
> No. 6: very heavy cloud (++++)

Exton's Test.—1. Filter the urine or remove the upper portion after centrifuging.

2. Mix equal volumes of urine and reagent in a test tube.

REAGENT

Sodium sulphate	.. ...	200 gm.
Sulphosalicylic acid		50 gm.
Water to make	.	1000 c c.

Dissolve the sulphate in 700 c c. of water with heat; allow to cool; then dissolve the acid without heat and dilute to 1000 c.c.

3. If cloudiness does not develop, albumin is absent and the reaction is negative.

4. Should cloudiness develop, warm slightly by passing through a flame. Do not boil. Any cloudiness which persists is due to albumin and constitutes a positive reaction.

5. If the urine is slightly cloudy and cannot be cleared, the reaction should

FIG. 83.—BOILING URINE.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore.)

be compared with a tube containing water and urine. A distinct difference will be noted if albumin is present.

6. Record as described above.

7. This test is quite sensitive and highly recommended for routine work.

Purdy's Test.—1. Fill a thin-walled test tube half full with urine.

2. Add about one-sixth its volume of saturated water solution of sodium chloride and 5 to 10 drops of 50 per cent acetic acid.

3. Mix well and boil the upper portion over a Bunsen burner (Fig. 83). A holder is unnecessary. Rotate or shake gently by heating to prevent cracking of the tube by condensation of steam.

4. A cloud (best seen against a dark background) denotes the presence of albumin (Fig. 84) and the results are recorded as described above.

5. This is a valuable routine test, as the addition of the sodium chloride raises the specific gravity and prevents precipitation of mucin. Bence-Jones protein may produce a cloud which disappears upon cooling.

6. This method may be adapted to the testing of a large number of samples at one time by using numbered tubes and placing in each about 5 c.c. of urine, 1 c.c. of sodium chloride solution and 5 drops of acetic acid, followed by mixing and placing in a boiling water bath for five minutes with the water above the level of the contents of the tubes.
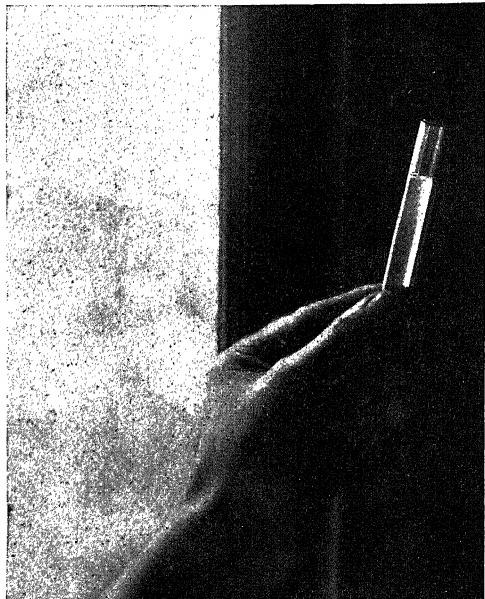
FIG. 84.—CLOUD OF ALBUMIN SEEN AGAINST A DARK BACKGROUND.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

Heat and Acid Test.—1. Boil about 5 c.c. of filtered urine in a test tube for one or two minutes. Hold with a clamp or a piece of filter paper folded around the neck.

2. Add 1 or 2 drops (no more) of concentrated nitric acid or 3 to 5 drops of acetic acid.

3. A white cloud now disappearing is due to earthly phosphates. Effervescence is generally due to carbonates from the food.

4. A very faint trace of albumin may appear only upon the addition of the acid. Larger traces appear upon boiling and may become heavier upon the addition of the acid. The addition of too much acid may dissolve faint traces of albumin and give a falsely negative reaction.

5. For the routine testing of a large number of samples by this method, use numbered test tubes and place in a boiling water bath for at least five minutes. Add the acid to each, mix gently and record the results as described above.

6. An advantage of this method is the fact that it allows a rough quantitative estimation of albumin from the volume of precipitate after standing overnight. Complete solidification amounts to 2 to 3 per cent albumin. Precipitates reaching one-half, one-third, one-fourth and one-tenth the height of the column of urine correspond roughly to about 1, 0.5, 0.25 and 0.1 per cent albumin.

7. Certain resinous acids may be precipitated by the acid but these may

be easily differentiated from albumin precipitate by reason of their solubility in alcohol.

Roberts' Test.—1. The test may be carried out by contact with urine in any of the following ways:

(*a*) Place a few c.c. of the reagent in a conical glass or test tube. Tilt and run clear urine from a pipet or medicine dropper down the side to give a sharp line of contact.

<div align="center">REAGENT</div>

Magnesium sulphate (sat. aq. sol.)............ 5 parts
Nitric acid (conc.)........................ 1 part

(*b*) Place urine in a horismascope and underlay with agent as shown in Figure 85B. This instrument is too fragile and too expensive for general use although very handy for office work.

<div align="center">A   B</div>
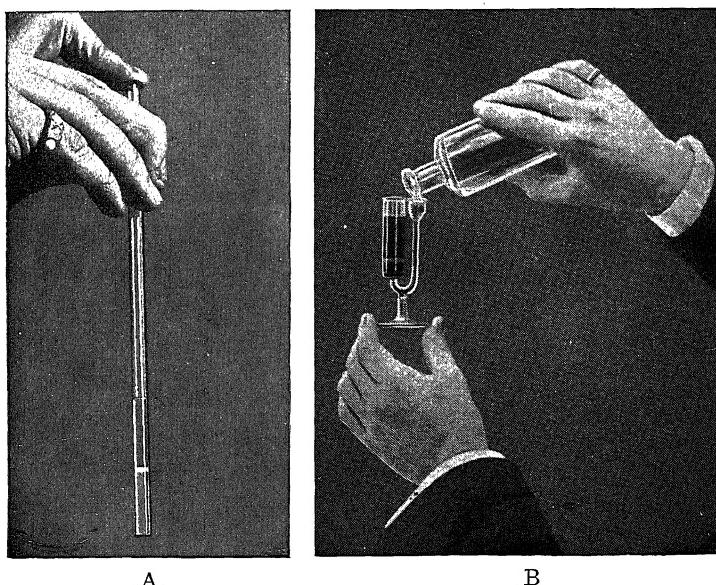
<div align="center">FIG. 85.—RING OR CONTACT TESTS.</div>

A. Boston's method. *B*. Horismascope method. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)

(*c*) Or immerse a pipet in the urine, wipe off the outside and immerse in the reagent, as shown in Figure 85A, according to Boston's method. Not as sensitive as (*a*) and (*b*).

2. If albumin is present, white rings appear at the line of contact, best seen against a black background at a distance of several feet.

3. Record as described above.

4. This test is much more satisfactory than the Heller ring test employing

nitric acid, as it is sensitive and does not form the secondary and confusing colored rings due to indican, bile pigments, or the oxidation of other organic constituents. It may, however, yield the secondary ring due to uric acid and urates and especially if the test is conducted in a conical glass. This ring is less sharply defined, broader and frequently situated above the albumin ring. Concentrated urines may occasionally exhibit a crystalline ring of urea nitrate, but this is easily distinguished from the "fluffy" ring of albumin.

QUANTITATIVE ESTIMATION OF ALBUMIN

Exton's Method.—1. Add 2 to 3 c.c. of reagent to an equal amount of urine in a scrupulously clean tube and mix thoroughly.

2. Allow to stand about five minutes and then heat, but do not boil the mixture, by passing the tube through a Bunsen flame several times.

3. Invert the tube several times and compare with the standard tubes furnished by Lehn and Fink, Inc., Bloomfield, N. J., representing 0.5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 milligrams protein for each 100 c.c. of urine. It may be necessary to dilute urine containing a large amount of albumin so that a comparison can be made with the standard tubes. The formula for the reagent accompanies the tubes.

Esbach's Method.—1. Fill with urine an Esbach-Quick albuminometer (Fig. 86) to the mark *U.*

2. Add reagent to the mark *R:*
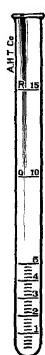
FIG 86 —ESBACH-QUICK ALBU-MINOMETER

REAGENT

Trichloracetic acid   .   ... 100 c.c.
Water . ... 900 c.c.

3. Close with a rubber stopper, invert slowly several times, and set aside in a cool place for eighteen to twenty-four hours.

4. Read off the results according to the markings on the tube which show albumin in grams per 1000 c.c.; to express the per cent, divide by 10.

5. The advantage of the new reagent over the former one of picric and citric acids is that the effects of temperature and specific gravity of the urine are reduced to a minimum. The latter reagent is, however, preferred by some and is prepared as follows:

Picric acid.   ....   10 gm.
Citric acid.   ....   20 gm.
Water   ..   ..   1000 c.c.

Tsuchiya's Method.—This method is generally regarded as superior to that of Esbach. If the urine is alkaline, acidify with a few drops of acetic acid and proceed in the same manner as in the Esbach method, using the following reagent:

Phosphotungstic acid.....	1.5 gm.
Hydrochloric acid (conc)	5.0 c c.
Alcohol (96 per cent)	95.0 c c.

Folin's Method.—1. Place 1 c.c. of urine in a test tube and add a 2 per cent solution of sulphosalicylic acid to exactly 25 c.c.

2. In four additional tubes of the same size place 1 c.c. of 0.1, 0.2, 0.3, and 0.4 per cent solutions of a stock albumin solution and add the reagent to 25 c.c.

3. Mix and let stand ten to fifteen minutes and compare.

4. A stock albumin solution of approximately 1 per cent strength is made by diluting sheep serum with 7 volumes of 15 per cent sodium chloride solution. This keeps well, and the other standards are made from it by dilution with 15 per cent sodium chloride solution.

DETECTION OF PROTEOSES

Principles.—Proteoses, particularly deuteroproteose and heteroproteose, have frequently been found in the urine under various pathological conditions. They are divided into two groups, namely, *primary* and *secondary*. The *primary proteoses* are precipitated upon half saturation with ammonium sulphate and the *secondary proteoses* upon complete saturation.

Procedure.—1. Acidify the urine with acetic acid and filter off any precipitate of nucleoprotein which may form.

2. Boil for several minutes.

3. Filter while hot to remove the albumin and globulin.

4. Test the filtrate by overlaying a saturated solution of trichloracetic acid. A white ring at the point of contact indicates the presence of proteoses.

5. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulphate.

DETECTION OF BENCE-JONES PROTEIN

1. Place the urine in a water bath with a thermometer and heat very slowly and gently.

2. Observe frequently. Turbidity will begin to occur at about 40° C. and precipitation will take place at about 60° C.

3. Now acidulate *very slightly* with acetic acid and raise the temperature to the boiling point (100° C.). The precipitate now partly or totally disappears.

4. Allow to cool and if Bence-Jones protein is present the precipitate will reappear.

5. If the test is positive it is advisable to confirm the results by one or both of the following tests:

(*a*) Precipitate the protein with nitric acid. This precipitate should disappear on boiling and reappear upon cooling.

(*b*) Precipitate the protein with alcohol and collect immediately by centrifuging. The precipitate should be soluble in water.

DETECTION OF DEXTROSE (GLUCOSE)

Principles.—1. Dextrose or glucose readily reduces the oxide of copper in alkaline solution. When the whitish-blue cupric hydroxide in suspension in an alkaline solution is heated it is converted into insoluble black cupric oxide, but if sugar is present this is reduced to insoluble yellow or red cuprous oxide.

2. A large number of tests have been devised on this principle for the detection of sugar in the urine but that of Benedict is recommended because of its sensitiveness, simplicity, and freedom from error. The qualitative reagent does not react with the normal sugar of the urine but detects increases above this level as low as 0.2 per cent. Furthermore, uric acid, creatinine, chloroform, formalin and other aldehydes do not interfere to such an extent as in the case of Fehling's test.

3. If albumin is present in large amounts, it may interfere with the precipitation of copper and should be removed by acidifying with acetic acid, boiling and filtering. Small amounts need not be removed.

Benedict's Test.—1. Place 5 c.c. of Benedict's *qualitative* reagent in a clean test tube.

BENEDICT'S QUALITATIVE REAGENT

Copper sulphate............	17.3 gm.
Sodium citrate......	173 0 gm.
Sodium carbonate (anhydrous)	100 0 gm.
Distilled water to make..	1000.0 c.c.

Dissolve the citrate and carbonate in about 500 c.c. of distilled water by boiling. Filter through paper.

Dissolve the copper sulphate in about 100 c.c. of water.

Add the copper solution slowly to the citrate and carbonate solution and stir continuously while adding.

Measure and add sufficient water to make the total volume 1000 c c. *Do not use for quantitative test.*

2. Add 0.5 c.c. of urine and mix thoroughly.

3. Boil thoroughly for two and one-half to five minutes; or place tubes in a boiling water bath for five minutes—a particularly convenient method when conducting a large number of tests at one time.

4. Allow to cool spontaneously.

5. If no sugar is present the solution will remain clear or show only a slight turbidity of a faint bluish color due to urates. If sugar is present a green, red, or yellow precipitate will form, the color depending upon the amount of sugar present.

6. Even 0.25 per cent glucose yields a large bulk of precipitate, filling the solution and rendering it opaque so that the test may be applied as readily in artificial light as in daylight.

7. The following scheme may be used for reporting (after Todd and Sanford):

+ (1) = *slight trace.*   No reduction is evident during boiling but appears upon cooling (greenish).

++ (2) = *trace.*   Reduction occurs after about one minute's boiling.

+++ (3) = *moderate.*   Reduction occurs after ten to fifteen seconds' boiling.

++++ (4) = *large amount.*   Reduction occurs almost immediately after adding urine to the boiling reagent.

Fermentation Test.—1. Place 15 c.c. of urine in a test tube and add a piece of fresh Fleischman yeast about the size of a pea; mix gently to emulsify the yeast.

2. Transfer to a fermentation tube; make sure the arm is free of bubbles of air.

3. Place in an incubator for a few hours.

4. A normal urine and a normal urine to which is added a pinch of glucose may be treated in the same manner as negative and positive controls respectively.

5. A positive reaction due to alcoholic fermentation is indicated by the collection of carbon dioxide gas in the arm.

6. If necessary, guard against gas production by bacterial fermentation by adding a pinch of tartaric acid (advisable if mixtures are incubated more than four hours).

7. By using the Einhorn saccharometer (Fig. 87), a *quantitative* test may be conducted, as the graduations on the arm indicate with fair accuracy the percentage of glucose present from 0.1 to 1 per cent.
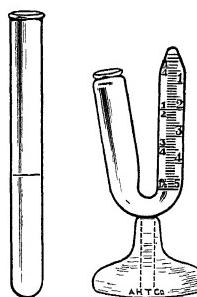
FIG. 87.—EINHORN SACCHAROMETER.
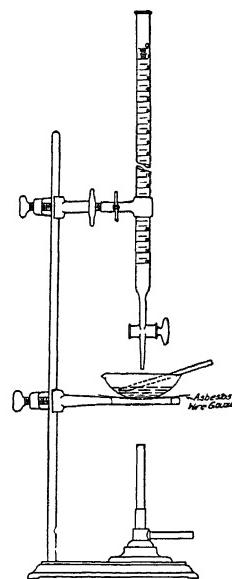
FIG. 88.—QUANTITATIVE ESTIMATION OF SUGAR IN URINE.

QUANTITATIVE ESTIMATION OF DEXTROSE

The tests given below are recommended for routine work.   More sensitive methods are described in Chapter XXXII.

Benedict's Method.—1. Dilute 10 c.c. of urine with 90 c.c. of water.

2. Mix and place in a buret (Fig. 88).

3. Place 25 c.c. of Benedict's quantitative reagent in a porcelain evaporating dish.

BENEDICT'S QUANTITATIVE REAGENT

Copper sulphate (pure crystallized)	18 gm.
Sodium carbonate (crystallized)	200 gm.
(or anhydrous sodium carbonate . 100 gm.)	
Sodium or potassium citrate (C.P.)	200 gm.
Potassium sulphocyanate (C.P)	125 gm.
Potassium ferrocyanide solution (5 per cent) .	5 c.c.
Distilled water to make.	1000 c.c.

With the aid of heat dissolve the carbonate, citrate and sulphocyanate in about 700 c.c. of the water and filter. Dissolve the copper in 100 c.c. of water and pour slowly in the other, stirring constantly. Add the ferrocyanide solution, cool and bring up to 1000 c.c. with distilled water. It keeps well.

4. Add to the reagent 10 grams of sodium carbonate crystals and a little pumice, talc or a few glass beads.

5. Apply heat to reagent until boiling.

6. While the reagent is boiling, run in the urine a little at a time but fairly rapidly, until a chalk-white precipitate appears and the blue color of the reagent begins to fade. Then add urine a drop or two at a time until all color disappears.

7. Note the amount of diluted urine used.

8. The reagent is so prepared that 25 c.c. are reduced by 0.05 gram of glucose. Therefore the amount of diluted urine used contains this amount.

9. Divide the amount of diluted urine by 10 to give the amount of undiluted urine carrying 0.05 gram of glucose. Divide 0.05 by the number of c.c. of undiluted urine to obtain the amount of sugar contained in 1 c.c. of urine; then multiply this number by 100 to obtain percentage or by the total number of c.c. in the twenty-four-hour specimen to obtain the number of grams voided. Example:

2480 c.c. of urine voided in twenty-four hours

8.2 c.c. urine 1 : 10 required to reduce 25 c.c. of reagent

$$\frac{8.2}{10} = 0.82 \text{ c.c. undiluted urine required}$$

$$\frac{0.05}{0.82} \times \frac{100}{1} = 6.1 \text{ per cent}$$

$$\frac{0.05}{0.82} \times \frac{2480}{1} = 151.2 \text{ gm. in twenty-four-hour specimen}$$

10. A short method to obtain the percentage is to divide 50 by the number of c.c. of diluted urine required to reduce 25 c.c. of the reagent. Example:

2480 c.c. voided in twenty-four hours
8.2 c.c. diluted urine required

$$\frac{50}{8.2} = 6.1 \text{ per cent}$$

11. If the urine contains but a trace of sugar it should be used undiluted and the percentage calculated as above. Example:

2870 c.c. voided in twenty-four hours
5.2 c.c. undiluted required

$$\frac{0.05}{5.2} \times \frac{100}{1} = 0.96 \text{ per cent}$$

Benedict's Test Tube Method.—1. Place 5 c.c. of Benedict's quantitative reagent in a clean test tube.

2. Add 1 or 2 grams of anhydrous sodium carbonate and an equal amount of pumice.

3. Heat to boiling over Bunsen burner.

4. While boiling run into tube from a 1 c.c. pipet, graduated in 0.1 c.c., undiluted urine until the last trace of blue has disappeared. The urine should be run in slowly and the solution kept boiling while the urine is being added.

5. As 5 c.c. of reagent are reduced by 0.010 gram of glucose, the amount of urine used contains this amount.

6. To obtain the percentage, divide 100 by the amount of urine and multiply by 0.010:

0.8 c.c. urine required

$$\frac{100}{0.8} \times \frac{0.010}{1} = 1.25 \text{ per cent}$$

7. Or divide 1 by the amount of urine to obtain the percentage:

$$\frac{1.0}{0.8} = 1.25 \text{ per cent}$$

8. If the urine contains a large amount of sugar, dilute 1:10 and calculate accordingly.

DETECTION OF LACTOSE

Principles.—Lactose is occasionally found in the urine of women during lactation and in patients who have been on an exclusive milk diet for a long time. It reduces copper solutions, although less actively than glucose.

Rubner's Test.—1. To 10 c.c. of urine add 3 grams of lead acetate (an excess).

2. Shake well and filter into a test tube.

3. Boil the filtrate for a few seconds; add 1 c.c. of strong ammonia and boil again.

4. If lactose is present, the solution turns brick-red and a red precipitate develops which is the criterion.

5. This test is not very sensitive but will detect lactose in about 0.3 to 0.5 per cent.

6. Dextrose (glucose) gives a red solution with a yellow precipitate.

7. Lactose does not ferment with yeast although bacteria may hydrolyze it into its constituents, glucose and galactose.

DETECTION OF PENTOSE

Principles.—Pentosuria may be *alimentary* and temporary, because of the ingestion of large amounts of pentose-rich fruits, or pathological (especially in diabetes). The pentose detected most frequently in the chronic form is arabinose. The color reaction is based upon the production of furfurol.

Bial's Orcinol Test.—1. First remove the dextrose by fermentation (see page 127). Filter.

2. Place 5 c.c. of reagent in a test tube and heat to boiling.

REAGENT

Hydrochloric acid (30 per cent).. .... 500 c.c.
Ferric chloride solution (10 per cent) .... 25 drops
Orcinol... .... 1 gm.

3. Remove from the flame and add the urine drop by drop (not exceeding 20 drops in all).

4. The appearance of a green color indicates presence of pentose.

5. This test is recommended because more accurate than the original orcinol test.

DETECTION OF ACETONE

Principles.—The detection of acetone in undistilled urine is based upon a color reaction with nitroprusside (Rothera's test) in which there is the formation of ferropentacyanide with the isonitro compound of the ketone or the formation of such an ion with the isonitro-anine derivative of the ketone. In the Frommer test the color is due to the formation of dihydroxydibenzoylacetone through the interaction of salicylaldehyde and acetone.

Rothera's Test.—1. To 5 or 10 c.c. of urine add about 1 gram of ammonium sulphate.

2. Add 2 or 3 drops of a freshly prepared 5 per cent solution of sodium nitroprusside.

3. Mix thoroughly.

4. Stratify with strong ammonium hydroxide.

5. If acetone is present, a permanganate color will develop at the line of contact.

A modification of this test is as follows:

1. Place 3 to 5 c.c. of urine in test tube.

2. Add 1 c.c. of reagent and mix thoroughly.

REAGENT

Ammonium nitrate	30 gm.
Sodium nitroprusside	2 gm.
Water (distilled)	80 c.c.

3. Overlay with strong ammonium water.

4. A purple or permanganate ring develops at the junction of the fluids if acetone or diacetic acid is present.

5. This test has the advantage of using urine without distillation. Diacetic acid, however, reacts quickly while acetone reacts more slowly and with less intense color, so that the two reactions grade into each other.

Frommer's Test.—1. To 10 c.c. of undistilled urine in a test tube add 3 c.c. of 40 per cent solution of sodium hydroxide.

2. Add 10 drops of a 10 per cent alcoholic solution of salicylaldehyde.

3. Mix and heat the upper portion to about 70° C. (do not boil) for five to ten minutes.

4. In the presence of acetone an orange color changing to deep red appears in the heated portion. A yellow to brown color is negative.

Wallhauser's Test.—1. Place 1 drop of Scott-Wilson reagent on an ordinary microscopic slide and place it over the mouth of the urine container to form a hanging drop, taking care that the reagent does not come in contact with the container.

REAGENT

Mercuric cyanide	1.0 gm.
Water	60.0 c.c.

Add a cooled solution of 18 grams of sodium hydroxide dissolved in 60 cc. of water. Transfer to a heavy glass jar and add 0.29 gram of silver nitrate dissolved in 40 c.c. of water while constantly stirring. Will keep for six months in a tightly stoppered dark bottle.

2. Allow to stand for two minutes.

3. Examine macroscopically.

4. If the reagent remains clear, the test has given a negative reaction. If cloudy, or a precipitate forms, a positive reaction is indicated.

DETECTION OF DIACETIC ACID

Principle.—The detection of diacetic (aceto-acetic) acid depends upon the production of a bordeaux red or violet red color with a dilute solution of ferric chloride.

Gerhardt's Test.—1. To about half a test tube full of fresh urine add a 10 per cent ferric chloride solution drop by drop until the phosphates are precipitated.

2. Filter.

3. To the filtrate add more of the ferric chloride solution or place a small amount of the reagent in a test tube and carefully overlay with filtrate for a ring or contact test.

4. If diacetic acid is present the solution will turn a bordeaux red color, but since a similar color may result from the presence of phenol, salicylates, antipyrine, sodium bicarbonate and other substances, it is necessary to repeat as follows:

1. To 5 c.c. of urine in a test tube add 5 c.c. of water and boil down to 5 c.c.

2. After cooling, add the ferric chloride as above.

3. Since boiling drives off diacetic acid, the development of the color indicates that it is due to other substances. If doubtful, apply the following test.

Lindemann's Test.—1. To 10 c.c. of urine in a test tube add 5 drops of 30 per cent acetic acid, 5 drops of Lugol's solution and 3 c.c. of chloroform.

2. Shake well and allow chloroform to settle.

3. If diacetic acid is present, the chloroform does not change color but becomes reddish-violet in its absence.

4. If the urine contains much uric acid, use double the amount of Lugol's solution.

DETECTION OF BETA-OXYBUTYRIC ACID

Hart's Test.—1. Dilute 20 c.c. of urine with an equal amount of water and add a few drops of acetic acid.

2. Reduce to one-half its volume by boiling to remove acetone and diacetic acid.

3. Dilute to 20 c.c. with water and place 10 c.c. in each of two test tubes.

4. To one tube add 1 c.c. of hydrogen peroxide and warm gently for one minute. Then allow to cool.

5. To both tubes add 10 drops of glacial acetic acid and 10 drops of freshly prepared concentrated sodium nitroprusside solution.

6. Mix thoroughly.

7. Overlay with strong ammonium water.

8. Allow to stand three or four hours.

9. A positive result is a purple ring in the tube treated with the peroxide, and none in the other.

DETECTION OF INDICAN

Principle.—The detection of indican (indoxyl potassium sulphate) by the test given below depends upon its decomposition and subsequent oxidation of the indoxyl set free into indigo blue and its absorption by chloroform.

Obermayer's Test.—1. Add to about 5 c.c. of urine in a test tube an equal volume of Obermayer's reagent and mix thoroughly.

REAGENT

Ferric chloride	2 gm.
Hydrochloric acid (conc. sp. gr. 1.19)	1000 c.c.

2. Heat until tube is warm.

3. Add 2 c.c. of chloroform and mix thoroughly by inverting, but avoid violent shaking.

4. Allow chloroform to settle.

5. If indican is present, the chloroform will be colored blue, ranging from a trace to a very deep blue, depending upon the amount present. The indican in normal urine may give a faint blue.

6. The urine of patients taking iodides may give a reddish-violet color which may obscure an indican reaction. By adding a few drops of concentrated sodium hypophosphite solution and shaking, the violet color will disappear, leaving the blue if indican be present. Occasionally, owing to slow oxidation, indigo red will form instead of indigo blue. This resembles the color given by iodides but does not disappear when treated with sodium hyposulphite.

7. Hexamethylenamine (urotropine) when taken by patients may prevent the reaction as likewise when it or formalin are added to urine as preservatives.

DETECTION OF BILE PIGMENTS

Principle.—The tests given below depend upon the oxidation of bile pigments by acids with the formation of a series of colored derivatives like biliverdin (green), bilicyanine (blue) and choletelin (yellow). Bilirubin is perhaps the most important pigment.

Rosenbach's Modification of Gmelin's Test.—1. Filter 100 c.c. or more of a urine through a small filter paper.

2. Remove the filter paper from the funnel and allow it to partially dry.

3. Touch the paper with a drop of old or yellow nitric acid.

4. If bile is present, a most marked spreading ring of rainbow colors with green on the outside will form.

Huppert's Test for Bilirubin.—1. To 10 or 15 c.c. of urine add a saturated solution of calcium chloride.

2. Filter.

3. Discard filtrate, and after perforating the filter wash precipitate into beaker with a small amount of alcohol acidulated with sulphuric acid.

4. Boil the solution.

5. If bilirubin is present, the solution will assume a bright emerald green color.

Schlesinger's Test for Urobilin.—It is claimed that urobilin is excreted as a chromogen, *urobilinogen,* which is changed by light into urobilin within a few hours after the urine is voided.

1. To 10 c.c. of urine in a test tube add a few drops of Lugol's solution to transform the chromogen into the pigment.

2. Add 10 c.c. of a saturated alcoholic solution of zinc acetate.

3. Mix and filter.

4. View the filtrate in sunlight against a dark background or with light concentrated upon it with a lens; a greenish flourescence indicates the presence of urobilin.

5. Bile pigment, if present, should be previously removed by adding one-fifth volume of 10 per cent solution of calcium chloride and filtering.

Ehrlich's Test for Urobilinogen.—Urobilinogen is a normal constituent and, as stated above, is converted into urobilin upon standing. It is increased whenever there is an excess of bilirubin formed through excessive destruction of erythrocytes, especially in pernicious anemia and malaria. When the liver cells fail to function properly there is an increase and for this reason the test has become of practical value as a *liver function test.*

A marked decrease or even total absence may occur in obstructive jaundice when the obstruction is complete or nearly complete. If the obstruction is only partial it may be normal or even increased.

1. Place 10 c.c. of urine in a test tube 15 millimeters in diameter. The urine should not be too cold. If very cold, allow it to stand at room temperature or gently heat to 65° F. to 80° F.

2. Add 1 c.c. of Ehrlich's reagent and mix.

<div align="center">REAGENT</div>

Paradimethylaminobenzaldehyde............. 10 gm.
Hydrochloric acid (conc.)....... .... 75 c.c.
Water .... 75 c.c.

3. Allow to stand for from three to five minutes, at the end of which time a cherry-red color appears if urobilin is present in abnormal amount. A light red and shades of pink appear when it is present in normal quantity. The examination for color should be made by viewing the contents through the mouth of the tube, holding it at a slight angle over white paper.

4. If no color appears the tube should be allowed to stand longer and then if still no color appears it should be heated and again examined before reporting an absence of urobilinogen.

This test is to some extent quantitative. With a little experience one can judge by the color whether there is an increase above normal.

5. A *quantitative* test may be conducted as follows:

(*a*) Place six test tubes (15 millimeters in diameter) in a rack. In them place the following amounts of urine:

No. 1: 10 c.c. 1:20
No. 2: 10 c.c. 1:30
No. 3: 10 c.c. 1:40
No. 4: 10 c.c. 1:50
No. 5: 10 c.c. 1:100
No. 6: 10 c.c. 1:200

The water used for diluting should not be too cold. If tap water is used it may be necessary to add enough warm water to bring it to about room temperature (between 65° F. and 85° F.).

(*b*) To each tube add 1 c.c. of Ehrlich's reagent (see above) and mix.

(*c*) At the end of *five minutes* examine the tubes by viewing through the mouth against a white background. Note the highest dilution which shows the slightest pink color. This is reported as positive 1:50, 1:200, etc.

When possible the readings should be made by daylight, as artificial light has a tendency to intensify the color. Highly concentrated urines may give a yellowish brown discoloration which has to be differentiated from the true pink reaction of urobilinogen.

(*d*) Bile pigment, if present, should be previously removed by adding 1 part of 10 per cent solution of calcium chloride to 4 parts of urine, and filtering.

Test for Urorosein.—This pigment does not occur in normal urine but may occur in various diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, and stomach disorders. It is excreted as a chromogen (indole acetic acid) which is transformed into the pigment upon treatment with a mineral acid:

1. Place 10 c.c. of urine in a test tube.

2. Add 5 c.c. of concentrated hydrochloric acid and a few drops of a 1 per cent solution of potassium nitrite.

3. Mix thoroughly.

4. A rose-red color indicates urorosein.

Ehrlich's Diazo Reaction.—The exact nature of the diazo substance or substances is unknown. It may be due to an increased excretion of urochromogen, alloxyproteic acid, oxyproteic acid, or uroferric acid. The reaction occurs in the urine in febrile disorders, especially typhoid fever, tuberculosis, and measles. Reactions more or less resembling it may occur after the administration of opium and its alkaloids, salol, creosote, phenol, the iodides, naphthalin and tannic acid.

The reagents are:

<div align="center">NO. 1</div>

Sulphanilic acid........................... 1.0 gm.
Hydrochloric acid (conc.).................. 10.0 c.c.
Water...................................... 200.0 c.c.

<div align="center">NO. 2</div>

Sodium nitrite........................... 0.5 gm.
Water.................................... 100.0 c.c.

<div align="center">NO. 3</div>

Strong ammonia water

1. Mix 10 c.c. of No. 1 with 0.1 c.c. of No. 2 in a test tube (Greene).
2. Mix and add an equal amount of urine.
3. Mix and carefully overlay with 1 or 2 c.c. of No. 3.
4. A positive reaction is indicated by a garnet (eosin pink to deep crimson) red color at the line of contact. Upon shaking a distinct pink color is imparted to the foam (essential feature). The color is a pure pink or red; any trace of yellow or orange is a negative reaction. A doubtful reaction should be considered negative.

DETECTION OF BILE ACIDS

Principles.—In Hay's test, advantage is taken of the fact that bile acids have the property of reducing the surface tension of fluids in which they are contained. Oliver's test depends upon the principle that a precipitate is formed of a protein (peptone) and bile acids.

Hay's Test.—1. Cool the urine by placing it in a refrigerator for several hours.

2. Upon the surface sprinkle a little finely powdered sulphur ("flowers of sulphur").

3. If the sulphur sinks at once, bile acids are present to the amount of 0.01 per cent or more. If the sulphur sinks only after gentle agitation, bile acids are present in 0.0025 per cent or more. If the sulphur remains floating, even after gentle shaking, bile acids are absent.

Oliver's Test.—1. Filter a small amount of urine (5 or 10 c.c.) until perfectly clear.

2. Acidify with acetic acid (not necessary if already acid).
3. Dilute with water until specific gravity is less than 1.008.
4. Place 2 c.c. in test tube.
5. Add 5 c.c. of reagent.

<div align="center">REAGENT</div>

Peptone.............................. 8.33 gm.
Salicylic acid....................... 1.12 gm.
Water containing 2 drops of acetic acid.... 1000.00 c.c.

6. A positive reaction is indicated by a milky turbidity.

DETECTION OF BLOOD

Principles.—The conditions in which blood occurs in the urine may be classified under *hematuria* and *hemoglobinuria*. In the former one is able to detect not only hemoglobin but the unruptured corpuscles as well (see microscopy,) whereas in the latter the hemoblogin alone is present.

The presence of blood is usually detected by the color of the urine, but the detection of traces requires microscopical and chemical examination. For the latter the usual tests for "occult blood" are required:

Benzidine Test.—1. Prepare a saturated solution of benzidine base (Merck's) by dissolving a knife point full in 2 c.c. of glacial acetic acid in a test tube. Warm if necessary.

2. Add an equal volume of 3 per cent hydrogen peroxide.

3. Add 2 c.c. of the urine and mix.

4. The appearance of a blue color indicates a positive reaction.

5. Set up a control, using water instead of urine.

As the reaction with urine may be sufficiently turbid to mask the color, the following test by Ruttan and Hardisty is recommended as being more satisfactory:

Orthotoluidine Test.—1. In a test tube mix 1 c.c. of reagent with 1 c.c. of urine and 1 c.c. of 3 per cent hydrogen peroxide.

REAGENT

Orthotoluidine.......................... 4.0 gm.
Glacial acetic acid....................... 100.0 c.c.

Dissolve. Keeps for a month without loss of delicacy.

2. In the presence of blood a bluish color develops (sometimes rather slowly) which persists for some time (several hours in some instances).

DETECTION OF HEMATOPORPHYRIN

Principle.—The pigment is precipitated with the phosphates, recovered, washed and prepared in solution in acid alcohol for spectroscopic examination.

Procedure.—1. To 100 c.c. of urine add 20 c.c. of a 10 per cent solution of sodium hydroxide.

2. Filter or centrifugalize off the precipitate.

3. Wash the precipitate with water and with alcohol.

4. Add 5 c.c. of alcohol and 5 to 10 drops of concentrated hydrochloric acid.

5. Dissolve, filter until absolutely clear and examine spectroscopically for the absorption bands of acid hematoporphyrin (Fig. 89).

6. An acetic acid test, which is much less reliable, consists in adding 5 c.c. of glacial acetic acid to 100 c.c. of urine and allowing the mixture to stand forty-eight hours. The pigment deposits in the form of a precipitate.
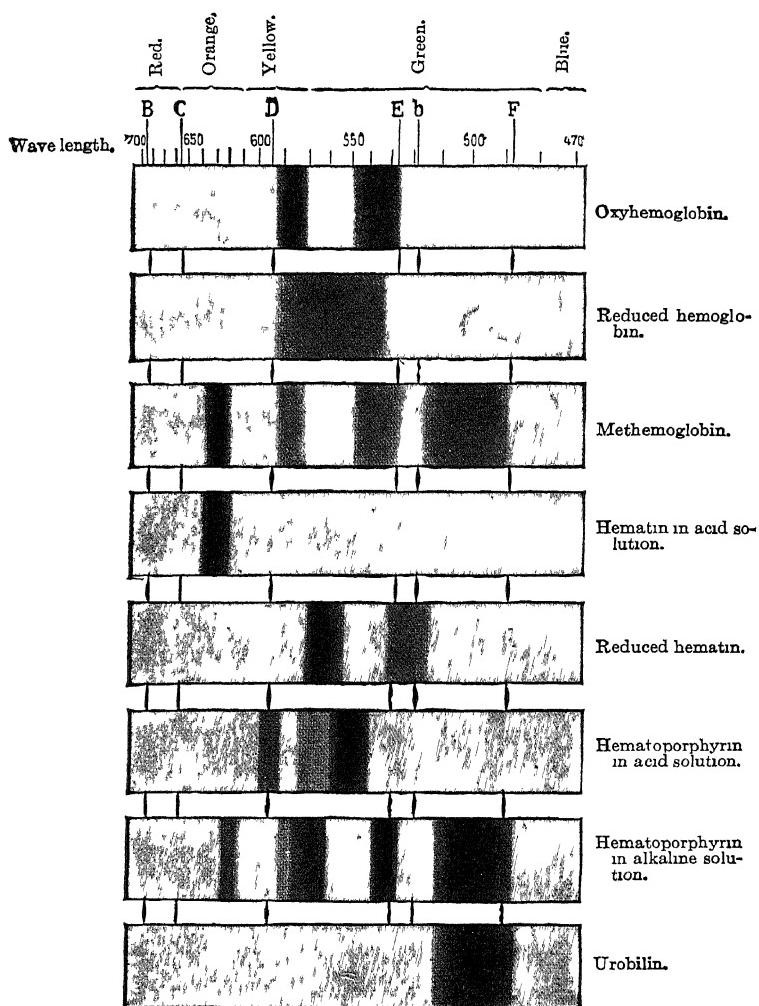
FIG 89—ABSORPTION SPECTRA.

(After Seifert and Muller.)

MICROSCOPIC EXAMINATION

General Recommendations.—1. As far as possible specimens should be examined within six hours after voiding.

2. Unless kept at a low temperature, twenty-four-hour specimens should have a preservative added.

3. Alkaline specimens cloudy with phosphates and obscuring other elements may be slightly acidified with dilute acetic acid to redissolve them.

4 Highly acid specimens containing heavy sediments of urates obscuring other elements may be slightly warmed to redissolve them.

5. If centrifuging is not employed, the sediment should be allowed to collect by gravity (preferably in a conical container) and examined before other tests are conducted.

6. Centrifuging, however, is required for the examination of small amounts of sediment and is advised routinely.

7. A qualitative method is described for the detection of organized and unorganized elements without reference to their number.

8. But since the findings vary greatly according to the technic employed, the quantitative method of Exton described below is highly recommended for routine use.

Qualitative Method.—1. Secure sediment by centrifuging at least 15 c.c. for three to five minutes or by allowing the urine to stand at least six to twelve hours in a cool place for settling by gravity (preferably in a conical container).
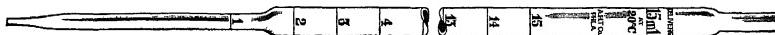
FIG 90—FOLIN-WU PIPET.

2. Remove a drop of sediment by means of a pipet and place on a slide. The pipet may be a piece of tubing drawn to a blunt point and fitted with a nipple. Eight may be prepared at one time by using slides of ordinary window glass, 4 by 8 inches, divided by painted lines into 8 compartments. The stage of the microscope may be extended by a wooden table, but this is not absolutely necessary.

3. Cover glasses are not essential for ordinary examination but are advisable for high-power examinations.

4. The examination must be completed before drying takes place.

5. Examine with low power and with oblique illumination obtained by swinging the mirror a little out of the optical axis. *Too strong illumination and too great magnification are common sources of error.*

Exton's Quantitative Method.—1. Pour well-mixed urine into a graduated centrifuge tube up to the 15 c.c. mark. Or plain centrifuge tubes may be used, etched with a line at 0.5 c.c. and a second line at 15 c.c.

2. Centrifuge for five minutes at 2000 revolutions per minute or equivalent.

3. Withdraw the upper 14.5 c.c. with Exton's special pipet; a Folin-Wu blood pipet (Fig. 90) fitted with a rubber bulb is satisfactory. The pipet should have a bore of at least 2 or 3 millimeters at the tip

4. Mix the remaining 0.5 c.c. of sediment or sediment with urine with the pipet by jiggling up and down and transfer a drop or two to an Exton urinalysis slide marketed by Bausch and Lomb (Fig 91). Two sizes are available for one and eight specimens.

5. Cover with a special cover glass 0.5 millimeter thick; an even spread of 0.1 millimeter is secured.

6. Allow to stand for a few minutes and examine. An apochromatic

system using an 8 millimeter objective and a 15 power (×) compensating eyepiece are recommended

7   Make counts of casts, pus cells, etc , in 10 high or 20 low power fields, and multiply by the factors given herewith according to the ocular (eyepiece)
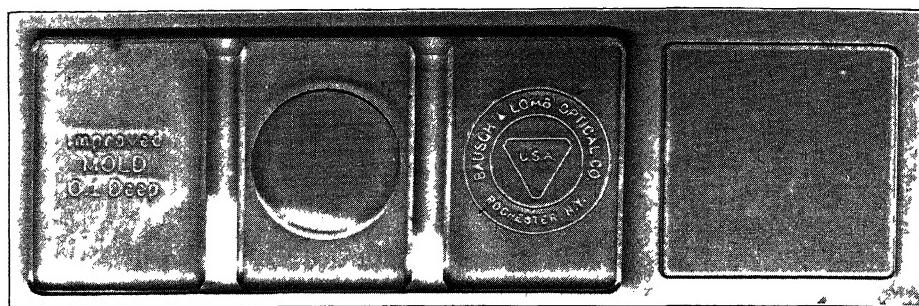
FIG 91 —EXTON URINALYSIS SLIDES
An eight cell slide with cover glass is also available

and objective (lens) used, *to count and report the number per 1 c c* of un-centrifuged urine ·

15× ocular and 8 mm objective (apochromatic) multiply by 1400
10× ocular and 4 mm objective (achromatic) multiply by 3100
10× ocular and 8 mm objective (achromatic) multiply by 700
10× ocular and 16 mm objective (achromatic) multiply by 170

Counting Chamber Method.—A counting chamber may be used, especially for counting leukocytes (pus cells)

1   The urine should be fresh, well shaken and examined as soon as possible after collection   Do not centrifuge

2   Fill a Thoma leukocyte pipet to the mark 1 with diluting fluid (5 per cent solution of glacial acetic acid), and to 11 with urine

3   Shake well   Discard 2 or 3 drops   Place a drop in a Fuchs-Rosenthal chamber as used for counting cells in spinal fluid   Adjust the cover glass

4   Wait five minutes for the cells to settle

5   With the 16 millimeter objective and 10×ocular, count all the cells in the entire ruling (9 sq mm)

6   Multiply by 100 and divide by 81 to give the number per c mm

7.   Multiply by 1000 to give the number per c c of undiluted urine
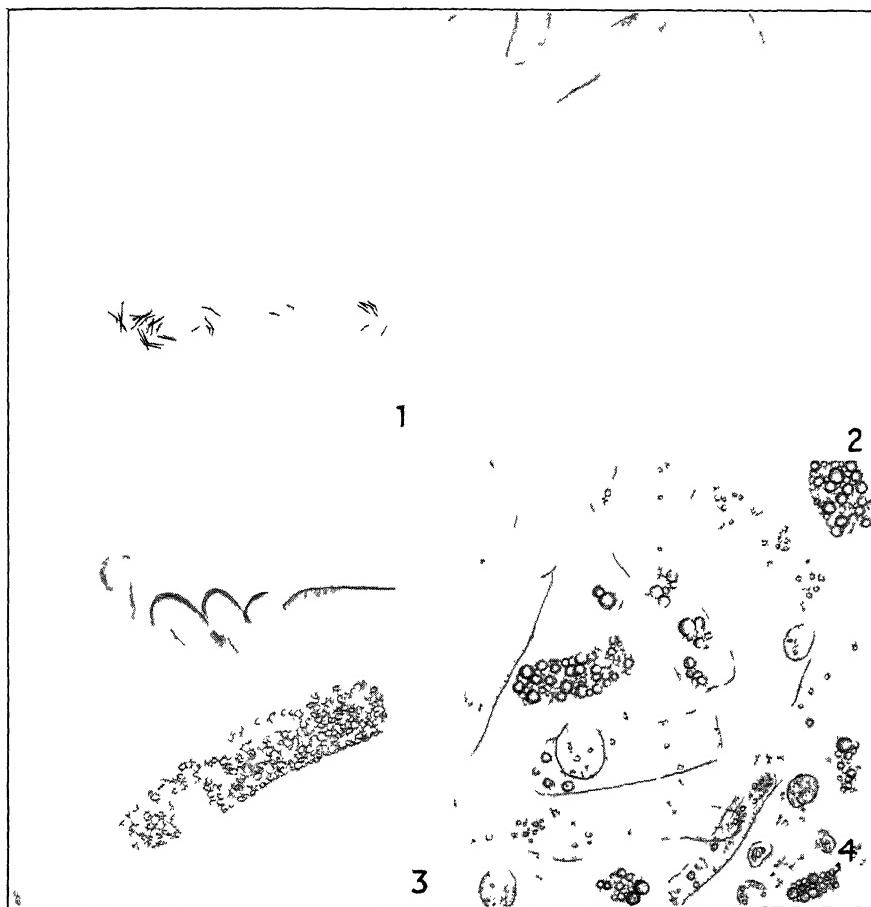
FIG 92—URINARY CASTS

1. Hyaline casts (after Riedert)   2 Hyaline and finely granular casts (after Todd and Sanford)   3 Waxy (colloid) and granular casts (after Riedert)   4 Granular and fatty casts (after Riedert)

What to Look for and Report upon

1 Casts of various varieties (Figs 92, 93)
    Hyaline casts (see 1 in Fig 92)
        Narrow
        Broad
    Waxy casts (see 3 in Fig 92)
    Fibrinous casts
    Granular casts
        Finely granular (see 2 in Fig 92)
        Coarsely granular (see 3 in Fig 92)
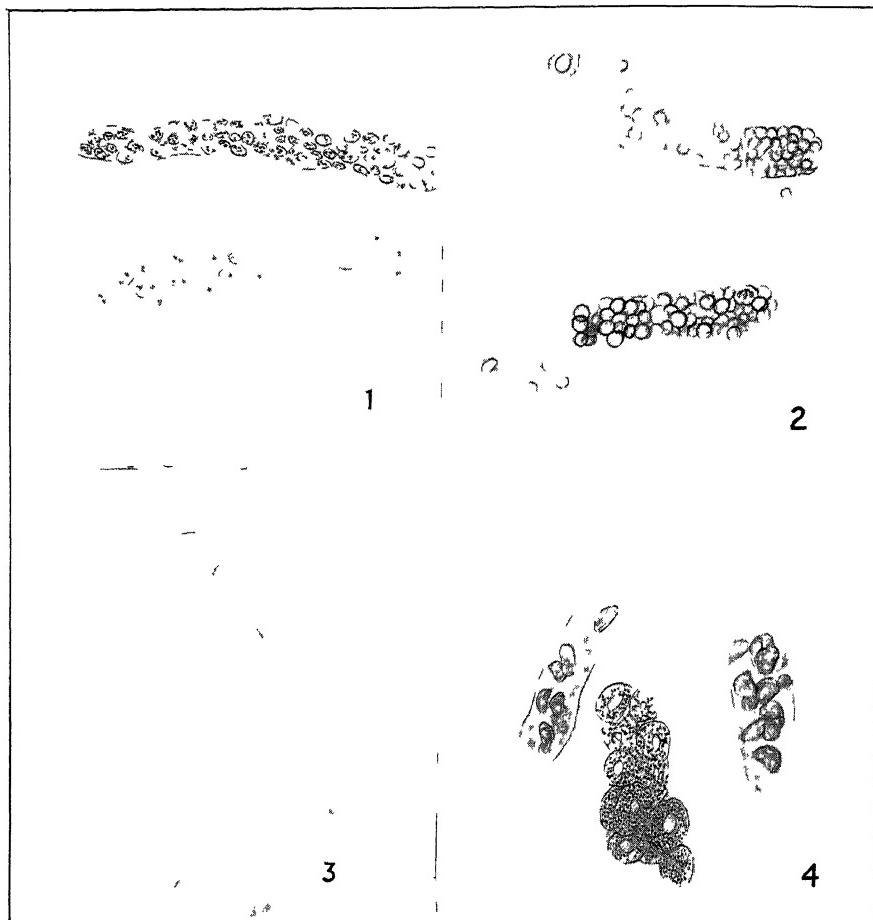
FIG 93—URINARY CASTS AND PSEUDOCASTS

1 Epithelial casts (after Riedert)   2. Blood casts (after Todd and Sanford)   3. Mucous threads and cylindroids (after Todd and Sanford)   4 Pseudocasts composed of swollen epithelial cells (after Riedert).

What to Look for and Report upon

1. Casts of various varieties—*Continued*

Fatty casts (see 4 in Fig. 92)

Casts containing organized structures

Epithelial casts (see 1 in Fig 93)

Blood casts (see 2 in Fig. 93)

Pus casts

Bacterial casts
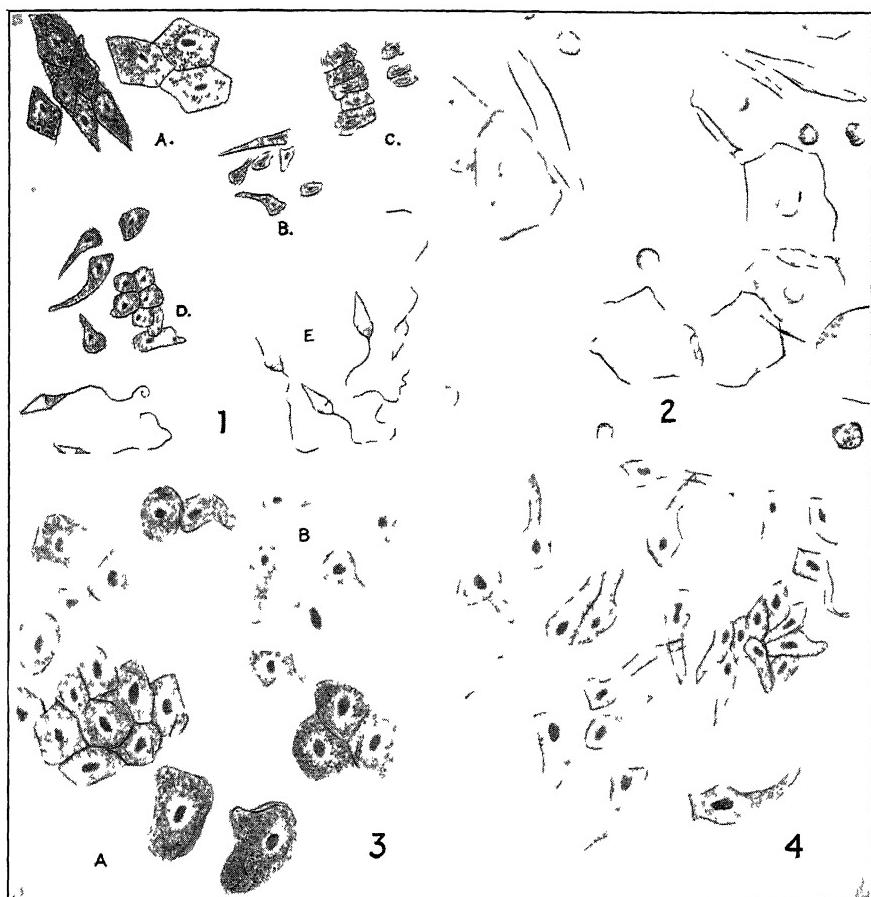
FIG 94—URINARY EPITHELIUM AND SPERMATOZOA

1 *A,* Vaginal epithelium, *B,* ureteral epithelium, *C,* renal epithelium; *D,* epithelium from pelvis of kidney; *E,* spermatozoa (all after Riedert). 2 Squamous epithelium and pus cells (after Todd and Sanford) 3 Epithelium from urethra (*B*) and bladder (*A*) (after Todd and Sanford). 4 Epithelium from pelvis of kidney (after Todd and Sanford)

What to Look for and Report upon

    2. Pseudocasts (see 4 in Fig 93)

    3. Cylindroids and mucous threads (see 3 in Fig. 93)

    4. Epithelial cells

        Vaginal (see 1 in Fig. 94)

        Renal (see 1 in Fig. 94)

        Ureteral (see 1 in Fig 94)

        Squamous (see 2 in Fig. 94)

        Urethra and bladder (see 3 in Fig. 94)

        Pelvis of kidney (see 4 in Fig 94)
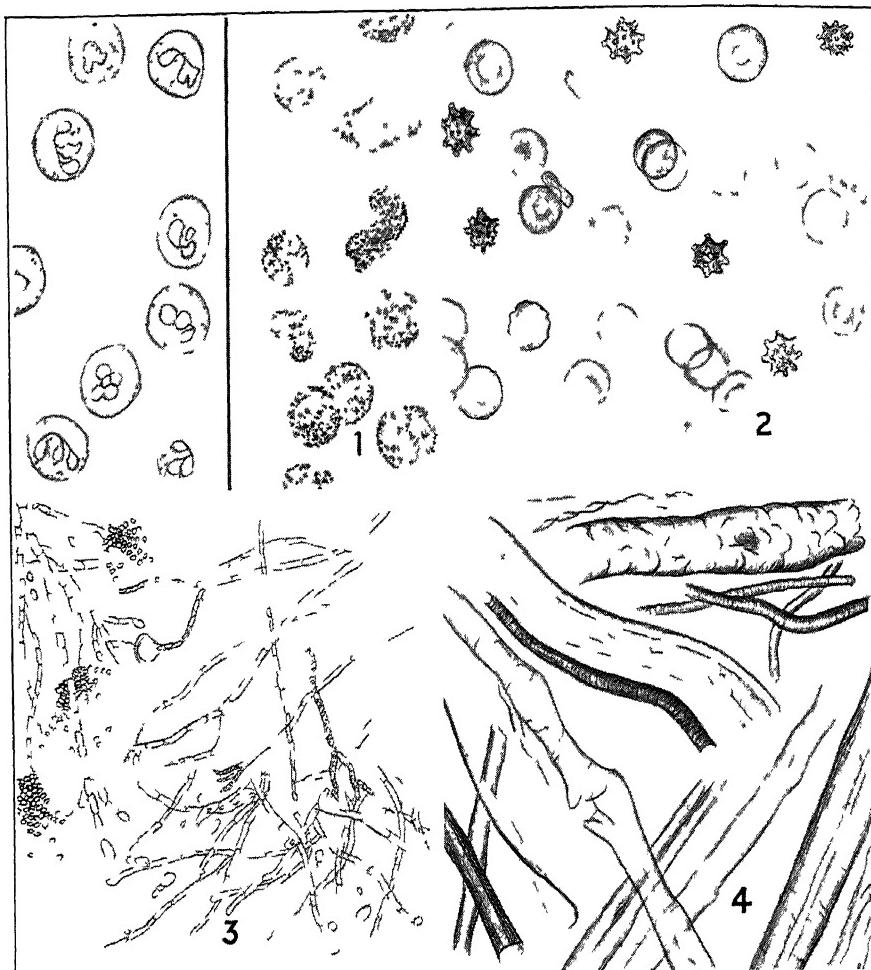
FIG 95—URINARY LEUKOCYTES, ERYTHROCYTES, MOLDS AND ARTEFACTS

1 Leukocytes (after Todd and Sanford)   2 Erythrocytes (after Todd and Sanford)
3 Molds (after Riedert)   4 Artefacts (after Riedert)

What to Look for and Report upon

5 Spermatozoa (see 1 in Fig 94)
6 Leukocytes (see 1 in Fig 95)
7 Erythrocytes (see 2 in Fig 95)
8 Molds and artefacts (see 3 and 4 in Fig. 95)
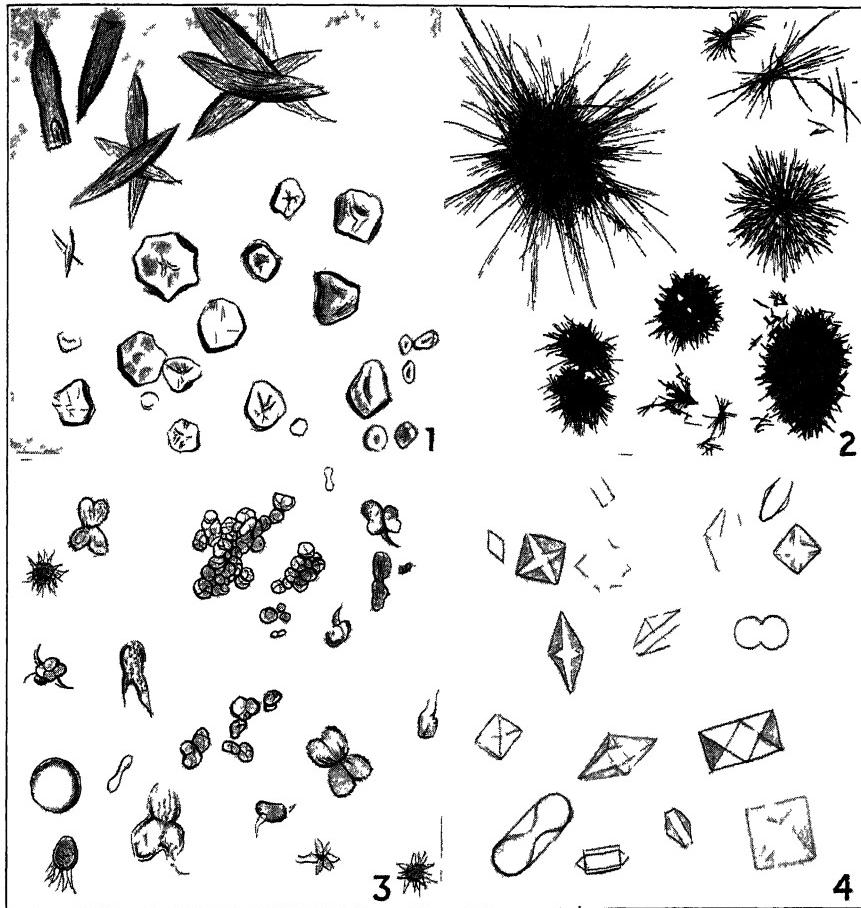
FIG 96—URINARY CRYSTALS

1 Uric acid (after Riedert)   2 Calcium urate (after Riedert)   3 Acid ammonium urate (after Riedert)   4 Calcium oxalate (after Todd and Sanford)

What to Look for and Report upon

9 Urinary crystals in acid urine

Uric acid (see 1 in Fig. 96)

Calcium urate (see 2 in Fig 96)

Acid ammonium urate (see 3 in Fig 96)

Calcium oxalate (see 4 in Fig 96)

Leucine and tryosine (see 4 in Fig 97)

Calcium sulphate (see 3 in Fig 97)
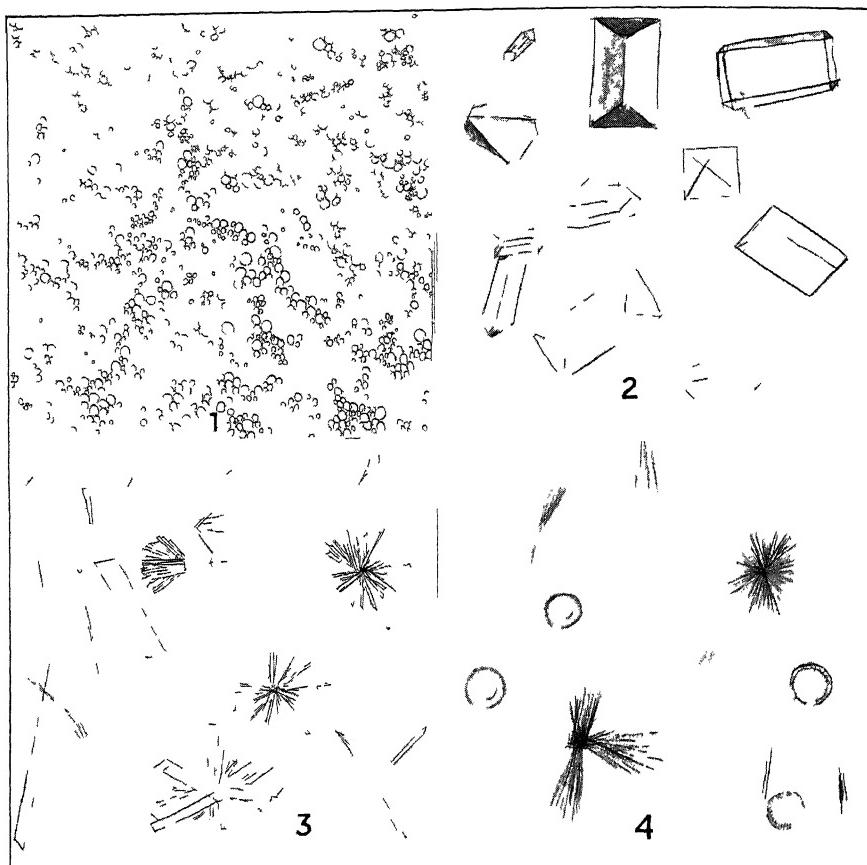
FIG 97—URINARY CRYSTALS

1 Amorphous phosphates (after Riedert)   2 Triple phosphates (after Todd and
Sanford)   3 Calcium sulphate (after Riedert)   4 Leucine and tyrosine (after Riedert)

What to Look for and Report upon

10 Urinary crystals in alkaline urine
Amorphous phosphates (see 1 in Fig. 97)
Triple phosphates (see 2 in Fig 97)

CHAPTER VII

METHODS FOR CONDUCTING KIDNEY FUNCTIONAL TESTS

PHENOLSULPHONPHTHALEIN TEST FOR KIDNEY FUNCTION

Principle.—A sterile solution of phenolsulphonphthalein is injected intramuscularly, and the amount excreted by the kidneys estimated one and two hours later by colorimetric determination.

Procedure.—MODIFICATION OF ORIGINAL ROWNTREE AND GERAGHTY PROCEDURE.—1 Have the patient pass urine, or catheterize if necessary. This specimen may be used for general examination.

2. Give 300 to 400 c c. (about 2 glasses) of water to promote secretion of urine.

3. Twenty minutes later inject intramuscularly exactly 1 c c. of the phenolsulphonphthalein solution (0.006 gram). Sterile solutions in ampules are on the market. If there is general edema interfering with absorption, inject intravenously.

4. Exactly one hour and ten minutes from the time of injection have the patient pass all urine (or catheterize), and label No. 1. The ten minutes represents the usual time elapsing between the injection and first appearance of the dye in the urine.

5. Exactly one hour later (two hours and ten minutes after the injection) have patient pass all urine (or catheterize), and label No. 2.

6. Measure Nos. 1 and 2 and record. If less than 40 c.c. each the results are not dependable.

7. Estimate the dye in each separately as follows:

(*a*) Place in a 1000 c c cylinder or graduate and add water to 800 c.c Add about 5 c.c. of a 10 per cent solution of sodium hydroxide, or sufficient to bring out the maximum purplish-red color, and add water to exactly 1000 c c. Mix well.

(*b*) Make the reading on each with a Duboscq, Bock-Benedict, Klett-Bio or other good colorimeter The standard should consist of 0 003 gram of phenolsulphonphthalein in 1000 c.c. of solution. The cylinder containing the standard may conveniently be placed at the 10 millimeter mark.

(*c*) Since the volume of each urine is the same as that of the standard, the percentage elimination in each may be calculated as follows:

Reading of urine : reading of standard :: 50 : X

8 The Dunning colorimeter (Fig. 98) is satisfactory for office work and consists of thirteen sealed ampules containing standard color solutions of different percentages, an open ampule in which the unknown specimen is placed, and a small box for comparing it with the standards. The colors remain good with but little fading for at least a year when kept in the dark.

9. Normally the first specimen shows 40 to 50 per cent elimination and the second 20 to 35, with a total of 60 to 75 per cent for the two hours.

PROCEDURE FOR KIDNEYS SEPARATELY.—1. Give 2 glasses of water about one half hour beforehand.

2. Place catheters in both ureters and then inject the dye *intravenously*.

3. Five minutes later collect urine from each side in test tubes carrying a few drops of 10 per cent solution of sodium hydroxide.

4. At subsequent intervals of fifteen or thirty minutes, collect urine in the same manner over a period of one half or one hour.

5. As soon as the dye is eliminated the urine turns pink in the test tube, but the time of its appearance is of less importance than the amount excreted.

6. Estimate the per cent excreted in each specimen separately by colorimetric determinations.

FIG. 98—DUNNING COLORIMETER

7. Normally the dye may appear in five minutes, but may be delayed in one or both kidneys as a result of reflex inhibition due to the catheters.

8. Normally *both kidneys together* eliminate 35 to 45 per cent in fifteen minutes, 50 to 60 per cent in the first half hour, and 65 to 80 per cent in the hour. When the catheters are removed, ascertain if urine has leaked past them into the bladder, as this accident confuses the results.

DETERMINATION OF PHENOLSULPHONPHTHALEIN IN THE URINE IN JAUNDICE

Principle.—Bile pigments are precipitated as barium salts and phenolsulphonphthalein determined in the filtrate in the usual manner. If such bile pigment is present, the amount of dye adsorbed by the precipitate is determined and the correction applied.

Procedure (Abramson).—When there is no correction for adsorption:

1. It is preferable to give the dye intravenously but intramuscular injection is almost as suitable.

2. Divide the two-hour specimen into two equal parts.

3. To one part add an excess (urine volume plus 50 c.c.) of saturated barium hydroxide. Dilute to 500 c c. or 1000 c.c. Filter. Compare a portion of the filtrate with standards. If the 500 c.c. dilution is used, the reading is direct. If 1000 c.c. is the dilution, the two-hour excretion is the reading
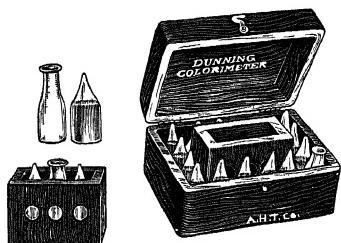
times 2. It is of course only necessary to catch a few c.c. of the filtrate. The dilution to 2 liters aids in colorimetric comparison.

4. If the total two-hour percentage of excretion of dye is normal or only 5 or 10 per cent below the lower limits of normal, the reading stands as the excretion of the dye and the excretion may be considered normal.

When correction for adsorption must be evaluated, continue as follows:

5. If the reading obtained is below normal, it must be determined whether the diminished excretion of the dye is actual or is due to adsorption. To the remaining half of the excreted urine, whose dye content has been determined, add 0.25 c.c. (for 500 c.c. dilution) or 0.50 c.c. (for 1000 c.c. dilution) of phenolsulphonphthalein. With intense jaundice and large urine volume, the latter is preferable. Redetermine, in this control, the percentage of dye.

6. The second reading in step 5 minus the first in step 4 gives the quantity of dye not adsorbed. For example, if the first reading (apparent two-hour excretion) had been 20 per cent, and if the remaining portions 0.25 c.c. of the dye had been added, the second reading, granting that none had been lost by adsorption, would be 20 per cent plus 50 per cent (0.25 c.c. dye to 500 c.c.), or 70 per cent. If the reading was 50 per cent instead of 70 per cent, it is evident that the added dye only gave an additional 50 per cent minus 20 per cent, or 30 per cent of color. Hence, three-fifths of the dye added was determined, and two-fifths lost by adsorption.

7. The final reading corrected by excreted dye is equal to the first reading divided by the fraction not adsorbed. For example:

$$\frac{20 \text{ per cent}}{3/5} = 20 \text{ per cent} \times 5/3 = 33 \text{ per cent}$$

TEST MEAL FOR RENAL FUNCTION

Principle.—This test is based upon the volume of night urine and variations in specific gravity of two-hour specimens during the day, when the patient is on a prescribed diet. Normally the urine excreted at different times during twenty-four hours varies markedly in volume and specific gravity. Pathologically the kidneys lose their adaptive powers to greater or lesser degree and consequently the urine which they secrete remains of almost uniform concentration from hour to hour.

Procedure (Mosenthal).—1. Upon the day of the test, and preferably also the day before, place the patient upon a full diet, such as the following: Breakfast of fruit, cereal, bread, butter, and tea, coffee, cocoa or water at 8 A.M.; dinner of soup, meat, vegetables, bread, butter, dessert and tea, coffee or water at noon; and supper of eggs, bread, butter, fruit, and tea or water at 5 P.M. Much latitude is allowable in the choice of foods, and in many cases the ordinary diet to which the patient is accustomed may be used. At least a pint of fluid—tea, coffee, water, etc.—must be taken at each meal; and no

food or liquid of any sort may be taken outside of these meals until after 8 o'clock the following morning.

2. Instruct the patient to empty his bladder immediately before breakfast. Collect specimens of urine at 10 A.M., noon, 2 P M., 4 P M., 6 P.M., 8 P.M., and finally at 8 o'clock the following morning. It is essential that the intervals be exact and that the bladder be completely emptied each time. Should the hour for the meals be changed, the times of collecting the samples of urine should be changed accordingly. The last of the two-hour specimens must not be collected less than three hours after the beginning of the evening meal.

3. Measure the night urine (8 P.M. to 8 A.M.) and take its specific gravity with an accurate urinometer.

4. Measure the six two-hour specimens and take their specific gravity, first making sure that they are all at the same temperature, since misleading figures may be obtained if some have been kept on ice and some at room temperature.

5. In health the urinary response is as follows:

(*a*) The night urine will be much less than the total day urine. It is usually 250 to 350 c.c., and will seldom exceed 400 to 500 c.c.; 750 c.c. is the maximum. Its specific gravity will usually be 1.018 or above.

(*b*) The highest specific gravity recorded for the two-hour day specimens will exceed 1.018 while the difference between the highest and the lowest will not be less than 8 or 9 points. If, for example, the most concentrated specimen has a specific gravity of 1.020, the most dilute will be 1.011 or less.

6. One or more of the following changes may be noted in impaired renal function:

(*a*) *Nocturnal polyuria.* The volume of the night urine exceeds 750 c.c. This is usually one of the first and most definite evidences of impaired kidney function. A volume between 500 and 750 c.c. is suspicious, and usually indicates impairment.

(*b*) *Low maximal specific gravity* of day urine, the highest of the two-hour specimens falling below 1.018.

(*c*) *Fixation of specific gravity*, that is, lessened variations in the specific gravities of the two-hour specimens. This is a very important sign of renal insufficiency. In marked cases the difference between the highest and lowest specific gravities may be only one or two points. As a rule, the level at which the specific gravity is fixed becomes lower as the functional impairment increases and the kidneys lose their ability to concentrate urine.

Fixation of specific gravity at a high level, near 1.018 or 1.020, may occur in acute nephritis, chronic parenchymatous nephritis, and passive congestion of the kidney, but is not necessarily an indication of disease, since it may occur when the patient has been taking insufficient water, or when there has been excessive loss of water through perspiration. Absorption and elimination of edema at the time of the test, leading to fixation of specific gravity at a low level, may also confuse the results.

CONCENTRATION AND DILUTION TESTS FOR RENAL FUNCTION

Principle.—These depend upon the concentrating and diluting powers of the kidneys as a test of their functional capacity. That for concentration should be done first and twenty-four hours allowed to elapse before beginning the dilution test.

Procedure (Volhard and Fahr) for Concentration.—1. Allow no fluids from the evening before the test until the test is finished, and no food between meals.

2. 8 A.M., breakfast: Dry cereal with sugar, syrup, or honey; no milk; one egg; toast or bread with butter.

3. 12 noon, dinner: Roast beef, steak, or chops; potatoes, boiled, baked, or riced; bread and butter; jam.

4. 5 P.M., supper: Two eggs; bread and butter; jam.

5. 8 A M. of same day: Empty bladder. Collect urine in separate containers every three hours thereafter until night, that is, at 11 A M., 2 P.M., 5 P.M., 8 P.M.; and collect all urine from 8 P.M. to 8 A.M. next morning in one container.

6. Note the quantity and specific gravity of each three-hour sample and of the twelve-hour sample and plot as a curve. Normally, the specific gravity of at least one sample should be 1.030, or at least 1.025.

Procedure for Dilution or Water Test.—1. Omit breakfast. For dinner and supper give the routine nephritic diet or the diet to which the patient has been accustomed. Permit one glass of water after supper.

2. 8 A.M.: Empty bladder and give 1500 c.c. water.

3. Collect urine in separate containers at 8:30, 9, 9:30, 10, 10:30, 11, 11:30 A.M. and 12 noon, eight specimens in all. Collect all the urine from 12 noon to 8 next morning in one container.

4. Note the quantity and specific gravity of each sample and plot as a curve. Normally, the total quantity voided should be 80 to 120 per cent of the intake (1200 to 1800 c.c.). The specific gravity of at least one sample should be as low as 1.003.

AMBARD'S COEFFICIENT AND MC LEAN'S INDEX TESTS FOR RENAL FUNCTION

Principles.—Ambard's coefficient is based upon determining the capacity of the kidneys for excreting urea by comparison of the concentration of urea in the blood with the rate of its excretion in the urine. The McLean index expresses the relationship by adopting 100 as the normal index on the basis that it falls in proportion to the degree of kidney insufficiency instead of rising as with Ambard's coefficient.

Procedure.—1. In the calculation of Ambard's coefficient the following

factors are taken into account, and they are represented in the formula by the symbols indicated:

$$D = \text{urea grams excreted in urine in twenty-four hours}$$

This is obtained as follows: (a) Give the patient 150 to 200 c.c. of water. (b) One half hour afterward have him empty his bladder and note the time of completion to the *minute*. (c) Discard this urine. (d) At the end of an exactly measured period, preferably two hours, have the patient again empty the bladder completely. (e) Measure this urine exactly and estimate its urea. (f) From this, calculate the number of grams of urea which would be excreted in twenty-four hours.

$$C = \text{urea grams in 1000 c.c. of urine}$$

This is calculated from the above estimation.

$$Ur = \text{urea grams in 1000 c.c. of blood}$$

Blood from a vein is taken in the middle of the period during which urine is collected, and the urea is estimated. One gram of urea nitrogen corresponds to 2.14 grams of urea.

$$Wt = \text{weight of patient, without clothing, in kilograms}$$

2. The above factors are combined in the following formula, in which the figure 70 is the standard normal body weight in kilograms and 25 is the standard amount of urea in grams per 1000 c.c. of urine.

$$\frac{Ur}{\sqrt{D \times \dfrac{70}{Wt}} \times \sqrt{\dfrac{C}{25}}} = \text{coefficient}$$

3. With normal kidneys a coefficient of 0.06 to 0.09 is obtained, regardless of how high the blood nitrogen may rise from diet or other causes. When the coefficient rises above 0.09 an impairment of the power of the kidneys to excrete urea is to be inferred.

4. McLean's index is based upon Ambard's coefficient and the formula is as follows:

$$\frac{D \times \sqrt{C} \times 8.96}{Wt \times Ur^2} = \text{index}$$

McLean has constructed a slide rule, by means of which it is possible with the given data to work out the index in a few minutes without calculation.

SODIUM BICARBONATE TEST FOR ACIDOSIS

Principle.—With normal individuals, administration of 3 to 5 grams of sodium bicarbonate by mouth will cause the urine to become alkaline. In conditions of acidosis, upon the other hand, very much larger amounts of bicarbonate may be given without bringing about this change. It appears that a large proportion of the carbonate is retained in the body to fortify the depleted alkali reserve of the blood and tissues. Only after the reserve is restored does the carbonate pass into the urine and change its reaction.

Procedure (Sellards).—1. Give the patient 5 grams of sodium bicarbonate, dissolved in a little water, by mouth, every two or three hours until the urine, voided before each dose, becomes neutral or faintly alkaline to litmus paper. The urine is thoroughly boiled before testing.

2. Tolerance of 20 to 30 grams of bicarbonate indicates a moderate grade of acidosis, which usually produces no clinical symptoms. Tolerance of 40 to 50 grams is noted in more marked grades which still do not lead to symptoms beyond dyspnea upon excretion. When the tolerance reaches 75 to 100 grams there may be very definite and serious clinical symptoms. In extreme cases the tolerance may reach 150 grams.

3. Since, however, large amounts of bicarbonate are not well borne by the stomach, it is well, in severe acidosis, not to push the administration until the urine becomes alkaline, but to discontinue as soon as the existence of marked acidosis is established.

CHAPTER VIII

METHODS FOR THE EXAMINATION OF SPUTUM

Principles.—1. In the great majority of instances sputum and bronchial secretions obtained by bronchoscopic drainage are submitted for bacteriological examination, especially for tubercle bacilli.

2. Valuable information is also to be sometimes obtained by careful macroscopical examination, although the finding of Charcot-Leyden crystals, Curschmann's spirals, Dittrich's plugs, etc., is not nearly as frequent as generally believed nor do they possess as much diagnostic value as surmised years ago before bacteriological methods came into general use.

OUTLINE OF COMPLETE EXAMINATION

The complete examination of sputum and bronchoscopic specimens may embrace the following:

1. Physical examination
   - (*a*) Consistency
   - (*b*) Color
   - (*c*) Odor
   - (*d*) Layer formation
   - (*e*) Reaction and specific gravity

2. Macroscopic examination aided by a hand lens
   - (*a*) Curschmann's spirals
   - (*b*) Dittrich's plugs
   - (*c*) Broncholiths or lung stones
   - (*d*) Bronchial casts
   - (*e*) Fibrin and coagulation

3. Microscopic examination of unstained specimens
   - (*a*) Elastic fibers
   - (*b*) Curschmann's spirals
   - (*c*) Charcot-Leyden crystals
   - (*d*) Pigmented or "heart-failure" cells
   - (*e*) Myelin globules
   - (*f*) *Actinomyces hominis* (ray fungus)
   - (*g*) Molds and yeasts
   - (*h*) Animal parasites

4. Microscopic examination of stained specimens
    (*a*) Tubercle bacilli
    (*b*) Pneumococci; streptococci; staphylococci; etc.
    (*c*) Spirochetes and fusiform bacilli
    (*d*) Pus
    (*e*) Eosinophils
    (*f*) Erythrocytes
    (*g*) Epithelium

5. Chemical examination
    (*a*) Albumin
    (*b*) Of *saliva* for nitrogenous substances and especially urea

COLLECTION

1. For tubercle bacilli, a twenty-four-hour specimen is recommended. For pneumococcus typing a single specimen may suffice. Special methods of collection for these and other bacteriological examinations are given in Chapter XV.

2. For tubercle and other general examinations sterile containers are unnecessary but they should be clean.

3. Morning sputum is recommended if single specimens are to be examined.

4. As a general rule it is advisable for the patient to wash the mouth and teeth before collection, especially of single specimens, to avoid extraneous contamination as far as possible.

5. A preservative like phenol or tricresol may be used for specimens to be examined for tubercle bacilli. Otherwise they should be avoided.

6. Patients should be instructed to distinguish between saliva and sputum. As far as possible the former and postnasal secretions should be avoided and deep bronchial secretions collected (for urea determination in saliva, see Chapter XXXI).

7. The container should be a small, wide-mouthed bottle or vial fitted with a tight stopper to prevent outside contamination and spilling, and to permit sterilization. Well-constructed paper sputum cups are acceptable. Paper napkins are unsuitable except for pneumococcus typing.

PHYSICAL EXAMINATION

1. If collected in a graduated glass, record the amount and whether or not layers are formed. Some sputa show a striking tendency to form three layers, especially in bronchiectasis, gangrene, and abscess of the lung.

2. Record the appearance as:
    (*a*) Mucoid (glairy, transparent and tenacious)
    (*b*) Purulent (pus or mucus and pus)
    (*c*) Serous (colorless or yellow; frothy)
    (*d*) Bloody (streaked; rusty; "prune-juice"; pure blood)

(e) Combinations of above

(f) Color (yellow; gray; greenish; rusty or brown; blackish; reddish or combinations)

(g) Odor (putrid; sweetish; cheesy, or none)

(h) Layers if kept in a tall glass for some hours

(i) Coagulation in case of bronchoscopic drainage specimens (complete; partial)

(j) Reaction (of little importance)

(k) Specific gravity (only upon special request)

      Mucoid sputa: usually 1.004 to 1.008

      Purulent sputa: usually 1.015 to 1.06

      Serous sputa: usually 1 037 and higher

MINUTE MACROSCOPIC EXAMINATION

1. Pour a portion of the sputum into a Petri dish or between two large panes of glass (former less messy and preferred) to give a thin layer.

FIG 99 —CURSCHMANN'S SPIRAL, SHOWING CENTRAL THREAD   (Wood)

2. Carefully examine against a black background with the aid of a hand lens

3. Pick out portions for microscopic examination.

4. Examine for:

(a) Curschmann's spirals, which are yellowish-white masses having a twisted appearance surrounded by mucus (Fig. 99)

(b) Bronchial casts (Fig. 100)

(c) Dittrich's plugs, which are yellowish-white bodies varying in size from a millet seed to a bean and having a very putrid odor

(d) Lung stones or broncholiths
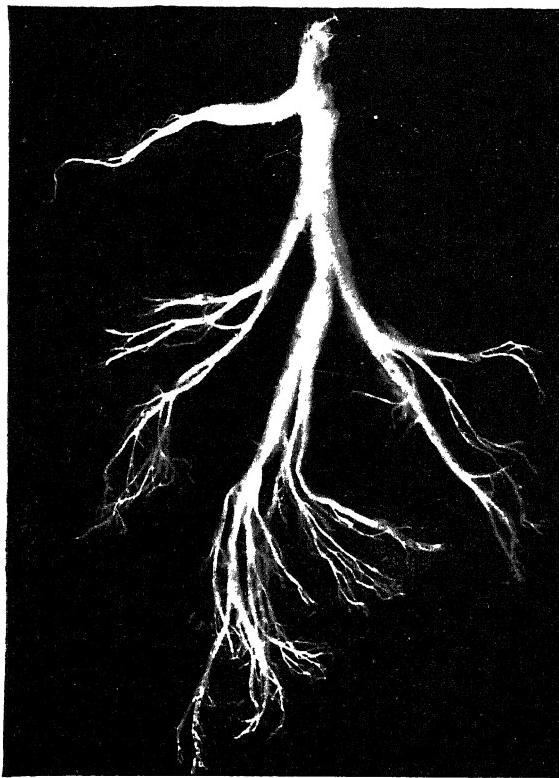
FIG. 100.—BRONCHIAL CAST   (Wood)

MICROSCOPIC EXAMINATION OF UNSTAINED SPUTUM

1. Carefully select suspicious particles and place on slides; cover with glasses.

2. Examine with low and high lens with the light well cut down as in the examination of urine sediment.

3. Examine for:

(a) Elastic fibers (Fig. 101)

(b) Pigmented cells ("heart-failure cells"): large epithelial cells containing hemosiderin, a brownish pigment. To demonstrate more clearly apply a drop of a 10 per cent solution of potassium ferrocyanide for a few minutes, followed by a drop of a normal solution of hydrochloric acid. Iron-containing pigment becomes a blue color (Prussian blue reaction)

(c) Curschmann's spirals

(d) Charcot-Leyden crystals (Fig. 102)

(e) Myelin globules, which possess little or no significance except that they may be confused with blastomyces (Fig. 103)

(f) *Actinomyces hominis* or "sulphur granules" (Fig. 104)

(g) Molds and yeasts as *Aspergillus fumigatus, Oidium albicans,* the fungus *Mycoderma,* etc.
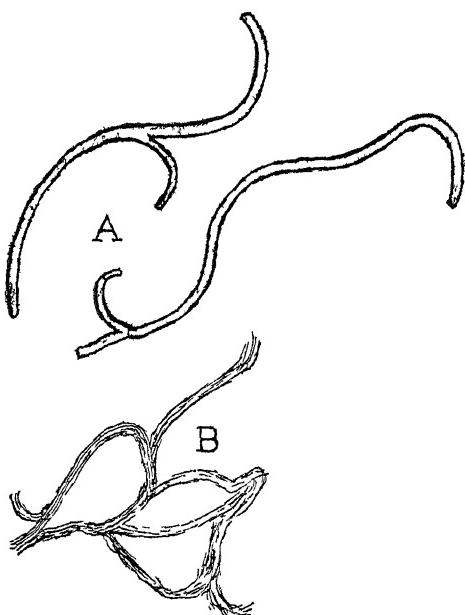
FIG. 101.—YELLOW ELASTIC TISSUE.

A Single fibrils, highly magnified *B.* Alveolar elastic tissue, lower power.   (Morris)

FIG 102 —CHARCOT-LEYDEN CRYSTALS. (Morris)
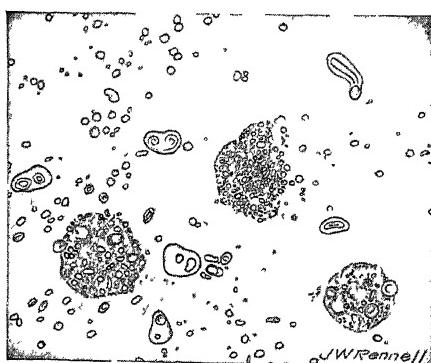
FIG. 103 —MYELIN GLOBULES   × 350.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)
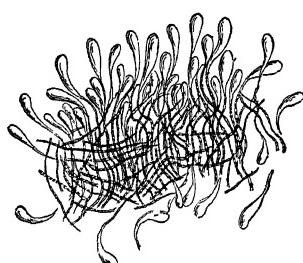
FIG. 104.—ACTINOMYCES HOMINIS, SHOWING CLUB-SHAPED EXTREMITIES TO THE RAYS   (Wood)

MICROSCOPIC EXAMINATION OF STAINED SPUTUM

1. For tubercle bacilli (Fig. E in Plate IV, facing p. 98), pneumococci, spirochetes, etc., see Chapters XXVIII and XXIX.

2. For eosinophils, pus, blood, etc., prepare thin smears on slides and stain with Wright's stain as described in Chapter VII for the staining of blood smears.

3. Examine for:
   (*a*) Eosinophils
   (*b*) Polymorphonuclear leukocytes (pus cells)
   (*c*) Erythrocytes
   (*d*) Kinds of epithelial cells

4. *Paragonimus westermanii,* called the "lung fluke," is a common parasite of man in Japan, China and Korea. It inhabits the lung, causing the formation of small cavities. Moderate hemoptysis is the principal symptom. Ova are readily found in the sputum (Fig. 105); the worms themselves are seldom seen, except postmortem. The worms somewhat resemble a coffee bean in size and shape. They are faintly reddish-brown in color, egg-shaped, with the ventral surface flattened, and measure 8 to 10 by 4 to 6 millimeters. The ova are thin-shelled, operculated, brownish yellow, and measure from 87 to 100 by 52 to 66 micra.

There are two intermediate hosts, a mollusk in which the cercariae are formed, and a fresh-water crab (a common article of food in Japan) in which they encyst. The encysted forms have also been found in fresh-water snails.

FIG. 105. — OVUM OF PARAGONIMUS RINGERI × 400. (After Emerson)

According to Ward, three distinct species have been confused under the name *Paragonimus westermanii:* the original form, *Paragonimus westermanii,* found in the tiger; the American lung fluke, *Paragonimus kellicotti,* thus far found only in cat, dog and hog; and the Asiatic lung fluke of man, *Paragonimus ringeri.*

CHEMICAL EXAMINATION

Test for Albumin.—1. Use fresh sputum, as decomposed specimens are unsatisfactory.

2. To 10 c.c. add 20 c.c. of a 3 per cent solution of acetic acid in water.

3. Shake vigorously and filter through paper.

4. Test the filtrate by the Esbach method for the quantitative determination of albumin in urine as described in Chapter VI.

5. In tuberculosis and pneumonia the albumin varies from 0.1 to 0.3 per cent or 1 to 3 grams per liter. In chronic bronchitis and asthma only traces are found.

Test for Occult Blood.—The benzidine or other methods described in Chapter XII for occult blood in feces may be employed.

Test of Mercury-Combining Power of Saliva for Nitrogenous Substances and Especially Urea (Hench and Aldrich).—REAGENT.—An accurately prepared 5 per cent solution of chemically pure mercuric chloride in distilled water.

COLLECTION OF SALIVA.—The mouth is well rinsed with water. Chewing of a small piece of paraffin or holding a small marble in the mouth will favor the flow of saliva, but this is not necessary. The saliva is collected in two portions of about 8 c.c. each. The first of these carries off food particles and epithelial débris and is discarded. The second is used for the titration. It need not be filtered.

METHOD.—1. By means of a pipet transfer 5 c.c. of the saliva to a small flask or beaker.

2. Add 5 per cent solution of mercuric chloride from a buret or pipet a few drops at a time, with constant stirring, until a drop of the fluid, when added to a drop of saturated solution of sodium carbonate on a white porcelain plate, gives a definite reddish-brown color. The color should appear within about three seconds. If it develops more slowly, the end-point is near, but not yet reached, and a few additional drops of the bichloride must be added

3. When the end-point is reached note the number of c.c. of mercuric chloride solution which have been added, and multiply by 20 to find the number of c.c. which would be required for 100 c.c. of saliva. Record this as the "mercury-combining index."

Hench has found the mercury-combining index in normal persons to lie between 30 and 50 for 100 c.c. of saliva. When there is retention of urea in the blood the index rises with the blood urea, although it lags a little behind The probable blood urea may be roughly calculated as follows:

$$1.43 \times \text{salivary index} - 34 = \text{probable blood urea in milligrams}$$
$$\text{for each 100 c.c.}$$

Example: Suppose the salivary index were 100. Then $1.43 \times 100 - 34 = 109$ milligrams urea in 100 c.c. of blood.

●

CHAPTER IX

METHODS FOR THE COLLECTION AND EXAMINATION OF STOMACH CONTENTS

INTRODUCTION OF THE STOMACH TUBE

1. The Rehfuss or Lyon tube is recommended.

2. If the patient is neurotic or has marked pharyngeal hyperesthesia, spray the fauces with a 2 per cent solution of cocaine hydrochloride.

3. Chill the tube in ice water. It may be lubricated with glycerin or liquid petrolatum, but these are undesirable.

4. The patient should be seated with the head tilted forward and the clothing protected with towels or an apron.

5. Place the tip of the tube on the tongue held well out, and pass it back to the throat. Then the patient is encouraged to swallow quickly upon removal of the fingers, while the tube is slowly fed into the mouth until the colored ring reaches the teeth. The patient should keep the lips closed and breathe deeply.

After slight discomfort in the pharynx and passage of the tube to the level of the cricoid cartilage, practically no discomfort is felt. If the patient is unable to swallow, he may be given a measured portion of the water of the test meal to swallow along with the tube, as this carries the latter to the stomach with a minimum of discomfort. The amount so given is, of course, considered in the final calculation.

It is sometimes advantageous to push the tube over to the side of the mouth, back of the teeth and passing behind the last molar tooth, just as soon as the tip has passed the esophagus. The patient should keep the mouth closed. This causes less reflex gagging and less chance of contaminating the stomach contents with mucus from the nose and pharynx.

6. If, now, the patient coughs or strains, the contents of the stomach will usually be forced out. If not, fluid may be obtained by alternate compression on the tube aided by the syringe. If unsuccessful, push the tube further in or withdraw it a few inches, since the distance to the stomach is not the same in all cases. If the tube becomes clogged it must be withdrawn, cleaned and reintroduced.

The easiest and least annoying way of removing the tube is to have the patient drink water, and, while swallowing, the tube can be quickly withdrawn.

METHODS OF EXAMINATION

1. Either of two methods may be employed: (*a*) The older consists in giving a Ewald breakfast and removing the stomach contents one hour later. (*b*) By the newer fractional method of Rehfuss, the tube is introduced into the fasting stomach, the residuum removed, a meal given with the tube *in situ* and fractions removed every fifteen minutes for one or two hours or longer.

2. If the older method is employed, the Topfer method of chemical analysis may be used; if the fractional method is used each portion is examined for free and total acidity, protein and any other tests decided upon.

3. The fractional method is recommended because it permits of a study of the gastric residuum, gives much more accurate information of the chemical and enzymic activities of the stomach and permits of an estimation of gastric emptying.

GASTRIC ANALYSIS BY THE FRACTIONAL METHOD

Procedure.—1. Removal of the residuum, feeding the test meal and removing samples every fifteen minutes for one to two hours.

2. Macroscopic examination of samples (see page 166).

3. Determination of total acidity of each sample (see page 167).

4. Determination of free hydrochloric acid of each sample (see page 168).

5. Testing for lactic acid (see page 171), especially when there is no free hydrochloric acid.

6. Testing for enzymes (see page 174), especially when there is no free hydrochloric acid.

7. Testing for protein (see page 173), especially when there is no free hydrochloric acid.

8. Testing for occult blood.

9. Microscopical examination of the residuum and of those samples containing bile.

REMOVING FRACTIONS OF STOMACH CONTENTS

Principles.—If properly carried out this constitutes one of the most valuable gastro-enterological examinations. Improperly and indifferently performed, it is of little value. The following classification represents a summary of the information which may be obtained by this method.

1. Measure of gastric work

  (*a*) Secretory-acidity and enzymatic determinations
  (*b*) Motor-emptying time of the Ewald meal or amount left in the stomach at the end of two hours following the Ewald meal

2. Indication of intragastric disease

  Addition to the gastric excretions of evidence of disease, such as: blood (microscopic or occult), pus, mucus (of stomach origin), desqua-

mated epithelium, bacterial colonies, tissue fragments and foreign bodies

3. Indication of extragastric disease
   (*a*) Extragastric type of acidity curve (indirect evidence)
   (*b*) Products of extragastric disease (direct evidence)
       (1) Swallowed pus, blood or mucus as indicative of lesion higher up
       (2) Persistent presence of bile in the stomach residuum or constant regurgitation of bile during the analysis is usually indicative of disease below the stomach disturbing the pyloric mechanism. Pathological products in this bile residuum as blood, pus, mucus, desquamated gall tract epithelium, cholesterin crystals, and numerous bile-stained organisms are additional confirmatory evidence

Procedure.—1. Patient should be instructed to take a motor meal, at 9 P M. on the night preceding the examination. A dish of rice and raisins, a meat sandwich and thirty raisins or a meal to include four stewed prunes will be satisfactory as the plan is to ingest some heavy cellulose which will be readily recognized in the gastric residuum the following morning.

2. The teeth should not be brushed on the morning of the examination to exclude any possibility of swallowing traumatic blood.

3. A gastroduodenal tube is passed to a point 22 inches from the lips as described above. Note should be made of the amount of gagging and retching which accompanies the passage of the tube. The reason for this observation is twofold. When there is considerable gagging and retching, bile is frequently regurgitated into the stomach. Recently regurgitated bile will be of lemon yellow tint and not a greenish turbid bile. Bile, after being in the stomach, becomes of a greenish turbid hue due to the action of the hydrochloric acid. Consequently the finding of clear lemon yellow bile in the stomach is usually due to regurgitation during the passage of the tube and is of no significance. As a rule patients who gag and retch considerably are of vagotonic make-up.

4. Extract all of the residuum with as little traumatism as possible. Measure the quantity of the residuum and save for examination.

5. Give the Ewald test meal with the tube *in situ, i.e.,* 35 grams of bread without the crust and 350 c.c. of water. Record the time either of the start or the finish of the meal. It makes little difference which, except that it should always be done in the same manner.

6. Any saliva which forms in the mouth after the meal is finished is to be expectorated into a basin and must not be swallowed. The irritation of the tube in some patients will cause an almost constant flow of saliva. If it is swallowed it will greatly reduce the stomach acidity. The amount of saliva expectorated in the two-hour period is measured and recorded. Normally

from 25 to 50 c.c. or less will be expectorated. If 200 c.c. or more are obtained, hyperptyalism is present, which may constitute further evidence of vagotonia. The saliva may be examined microscopically and chemically for enzyme content to eliminate any disease of the salivary glands if there are pointings in that direction.

7 An extraction should be made every fifteen minutes. An amount approximating 10 c.c. should be withdrawn with each extraction in order that a sufficient quantity of juice will be available for any special examinations which may be desired. In making the extractions, as little traction on the syringe as possible is desirable. If considerable suction is made on the syringe when the tube is collapsed, the mucosa will be traumatized and occult or gross blood will be the result. If such traumatism has been caused it is well to note the fact so that the occult blood reactions may be properly interpreted. It will be remembered that one of the important points to be learned from the fractional examination is the motor power of the stomach. With this in mind there are two methods of terminating the examination:

The method recommended is to withdraw specimens every fifteen minutes for a period of two hours from the time of ingestion of the meal. Then at the end of two hours the stomach is emptied by the syringe and the amount of chyme left in the stomach is measured and recorded. A food residue of much over 5 c.c. at the end of the two-hour period is indicative of delay in emptying. The amount of the residue will indicate the degree of hypomotility After the stomach has been emptied with the syringe, a lavage of 250 c c. of water should be made. Allow that amount of water to run in and out. This will give positive information as to the emptiness of the stomach and will act as a check on the emptying of the stomach with the syringe.

The other method consists in withdrawing fifteen-minute extractions until the stomach is completely empty to get the measure of motility of the stomach. This procedure takes considerably longer in the hypomotile cases and is not necessary if one gauges the amount of food remaining in the stomach at the end of two hours and learns to compute the degree of motor impairment. The first procedure also has the advantage that it requires less time and is less tiring to the patient.

8. The following data should be recorded concerning each sample which is extracted:

(a) A record is made of the amount of secretion which is withdrawn at each extraction. It is well not to remove more than 10 c.c. each time in order to allow the bulk of the food to be acted upon by the stomach. At the end of two hours the stomach is emptied. In order to be sure that the stomach is empty the patient should be told to assume various positions, aspiration being done in each position, i.e , on the back, stomach, right side and left side. The amount withdrawn at this last extraction is also recorded. The sum of the amounts withdrawn throughout the examination subtracted from the amount

of liquid given with the meal will represent roughly the amount of fluid which has passed through the pylorus in two hours. This, of course, does not allow for the amount of juice secreted.

(*b*) A rough estimation of the amount of food withdrawn with each extraction should be made. This will give some idea of the motility of the stomach. If after an hour or an hour and one half no food is obtained with the extraction, hypermotility must be suspected, providing the tube is in the right position. If a residue at the end of two hours shows more than 10 c c. of food at the bottom of the glass, the stomach motility is impaired. In the presence of an abnormal amount of food at this time, it is often impossible to extract all of the stomach residue. For this reason it is well to routinely lavage the stomach with 250 c c. of water after the last extraction has been made. The amount of food residue in the return wash is recorded. Finding more than 10 c c. of food in the return wash and the last extraction combined indicates hypomotility. The more above 10 c.c. the greater the degree of hypomotility or obstruction. This is one of the most important steps in fractional gastric analysis. Too often in routine hospital and office work the examiner is satisfied with a report of the acidity alone, which constitutes only a minor part of the information which may be obtained if this test is carried out properly. This method of testing the emptying time of the stomach is just as accurate as the six-hour barium x-ray meal and can be carried out often when an x-ray examination is not feasible. It checks with the x-ray method in the severe cases of obstruction and is a more refined and accurate method where the delay is slight.

(*c*) A record is made of the color of each extraction. This is of particular importance from two standpoints. Bile, regurgitating into the stomach, gives the stomach juices a yellow or greenish tint. The amount of bile should be recorded. It should be gauged by the intensity of the color, and recorded as plus 1, 2, 3 and 4. A small amount of bile may be found in one extraction in the normal stomach. But the presence of bile in four or five samples or at several consecutive examinations is indicative of disease. A regurgitation of bile and alkaline duodenal and pancreatic juices into the stomach is nature's way of neutralizing excessive acidity. It will frequently be found in cases of hyperchlorhydria. Anything which disturbs the pyloric mechanism, as disease of the gallbladder or duodenum, may cause an antiperistaltic movement of the duodenum and stomach with a resultant appearance of bile in the stomach. A rigid pylorus, adhesions of the pylorus or duodenum, or pancreatic disease may also cause regurgitation of bile. The appearance of bile in the fasting stomach and also evidence of freshly regurgitated bile in most of the samples withdrawn after the meal has been taken, are indicative of disease in many cases Of all the causes to be considered, gall tract disease must be considered as the most likely.

Gross blood in the stomach often gives the contents a diffuse brown tint. A sufficient amount of blood to cause its macroscopic appearance in the stomach juice means serious disease of or about the stomach, unless it has been swallowed.

MACROSCOPIC EXAMINATION

1. The *amount* of juice in the fasting stomach should not exceed 50 c c. It usually varies from 15 to 40 c.c. normally. An increase above 50 c.c. means either hypersecretion, hypomotility or obstruction. There should be no food in the eight-hour fasting stomach. It should be remembered that a very nervous patient may swallow very large amounts of saliva during the passage of the tube and greatly increase the amount of the residuum in that manner. However, it is not difficult to differentiate mouth mucus from stomach juice.

2. *Bile* in the fasting residuum, if found repeatedly and in large quantities, is indicative of some disease if the patient is "tube broken." The commoner conditions to be thought of in this connection are hyperchlorhydria, gall tract disease, duodenal ulcer, duodenitis and rigid pylorus from stenosis or adhesions. This should not be confused with the recently regurgitated bile due to the retching in the taking of the tube. Such recently regurgitated bile is lemon yellow in color and forms a yellow foam when shaken On the other hand, bile which has resided in the stomach for a considerable period is distinctly green and turbid. Some observers claim that bile is normally found in the gastric residuum. It is occasionally present but if it is present repeatedly, as a rule, there is some disease to account for it.

3. *Mucus,* if of stomach origin and present in considerable quantity, is indicative of catarrhal inflammation of the stomach. It is important, in this connection, to be able to differentiate stomach mucus from swallowed mucus. If it originates in the stomach it is flaky with particles suspended in the gastric juice. On the other hand, swallowed mucus is generally in large stringy masses and floats on the top of the gastric juice.

4. *Pus,* present in sufficient quantities to be suspected, is very rare. Such a finding would point toward acute or chronic diffuse suppuration of the stomach, abscess of the stomach or rupture of an abscess into the stomach (subphrenic, retroperitoneal, pancreatic, biliary, liver or splenic).

5. *Odor* of the gastric residuum is not usually of much importance. In cancer and severe catarrhal gastritis, a pungent disagreeable odor may be experienced. Colon bacillus infection of the stomach, of course, has a characteristic odor but it is very rare. A very unpleasant more or less characteristic sour odor is present in fermentation of the stomach contents.

6. *Blood,* if grossly recognized in the fasting residuum, is of the utmost importance. More than 50 per cent of all cases of cancer of the stomach will show small quantities of blood in the stomach. As a rule it is changed by the stomach juices and somewhat resembles coffee grounds. The average cancer

does not bleed profusely but it bleeds constantly, hence there is considerable changed blood in the stomach at any given time. Gross blood is not as common in ulcer of the stomach. Occasionally, however, it will be present in very large quantities and is often bright red in color. Esophageal varix, sclerosed stomach vessel, erosions and severe infections also give rise to free blood in the stomach but more rarely. Duodenitis, duodenal ulcer, pancreatic or biliary carcinoma may cause the appearance of free blood in the stomach. Injury to the mouth or esophageal mucosa in swallowing the tube and blood from the lungs and nose should be borne in mind.

7. *Food* in the fasting stomach is indicative of atony, dilatation, or ptosis of the stomach or, what is more likely, pyloric stenosis, pyloric or duodenal adhesions, pylorospasm, mass at or near the pylorus or pressure from without. The amount of food present and the constancy of its presence will help to decide whether one of the more serious causes is the most likely.

DETERMINATION OF TOTAL ACIDITY IN FRACTIONAL ANALYSIS

Principle.—Since the indicator phenolphthalein reacts with hydrochloric acid, combined acid (protein salt of hydrochloric acid), organic acids and acid salts, the method of titration determines the *total* acidity of the specimen.

Procedure.—1. Filter the specimens, if necessary, through cotton or fine-mesh cheesecloth.

2. Place 5 c.c. of each into small low-form porcelain evaporating dishes numbered and arranged in a row.

3. Dilute each with 10 c.c. of distilled water.

4 Add 2 drops of a 1 per cent alcoholic solution of phenolphthalein to each. This produces no essential change in color (see *A* in Plate V).

5. Titrate No. 1 with N/10 sodium hydroxide until a faint pink color is obtained and persists for about two minutes (see *A'* in Plate V). Record the amount required.

6. Return No. 1 to its place and titrate No. 2 and additional samples in the same manner.

7. Multiply the amount of N/10 sodium hydroxide required by 20 to obtain the amount of N/10 sodium hydroxide required to neutralize 100 c.c. of stomach contents. Repeat with No. 2 and additional samples and plot the results.

8. This procedure is subject to slight error due to the rapid addition of the sodium hydroxide but it is uniform, speedy and sufficiently accurate.

9. If 5 c.c. amounts of gastric juice cannot be used, place 1 c.c. amounts in evaporating dishes and add 4 c.c. of distilled water to each. Titrate with N/20 sodium hydroxide and multiply the amount by 50 to obtain the amount of N/10 required for the neutralization of 100 c.c. of gastric juice. Or the titration may be conducted with N/100 sodium hydroxide and the amount multiplied by 10.

DETERMINATION OF FREE HYDROCHLORIC ACID IN FRACTIONAL ANALYSIS

Principle.—The addition of dimethyl-amino-azobenzene (Topfer's reagent) gives an orange-red color if the acid is present which turns to a yellow upon neutralization with sodium hydroxide. It is subject to the error of an uncertain end-point.

Procedure.—1. Place 5 c c. of each of the strained samples into numbered porcelain dishes.

2. Add 10 c.c. of distilled water to each.

3. Add 2 drops of a 0 5 per cent solution of dimethyl-amino-azobenzene in 95 per cent alcohol to each.

4 If free hydrochloric acid is present a red or orange color develops (see C in Plate V).

5. Titrate No. 1 with N/10 sodium hydroxide until the orange-red color disappears (see C' in Plate V) and record the amount used.

6. Repeat with the other samples.

7. Multiply the amount of N/10 required for each sample by 20 to obtain the amount of N/10 required for 100 c c. of each. Plot the results into a curve.

8. If 5 c.c. amounts of gastric juice cannot be used, place 1 c.c. amounts in evaporating dishes and add 4 c c. of distilled water to each. Titrate with N/20 sodium hydroxide and multiply the amount by 50 to obtain the amount of N/10 required for the neutralization of 100 c.c. of gastric juice. Or the titration may be conducted with N/100 sodium hydroxide and the amount multiplied by 10.

CURVES OF TOTAL ACIDITY AND FREE HYDROCHLORIC ACID

1. **Normal Curve.**—The apex of the curve is reached at from sixty to ninety minutes after the Ewald meal and a return to within 20 degrees of the fasting acidity occurs in two hours. The apex values average: free hydrochloric acid, 45 to 55; total acidity, 55 to 65. This is the most frequent type of curve found in normal individuals, but there is often a wide deviation from this normal contour in otherwise normal people.

2. **Hyperchlorhydria Curve.**—The contour of this curve conforms to the normal curve except that the acid values are much higher. The fasting acidity will be free hydrochloric acid, 40; total acidity, 55 or higher. The apex of the curve will show: free hydrochloric acid, 75; total acidity, 90 or higher. This type of curve is occasionally found in normal individuals, but more commonly in cases of early or incipient gastric ulcer.

3. **Stepladder Curve.**—Described by Lyon and Best and supposed to be of serious diagnostic import. According to these observers it is only seen in cases of active ulcer and usually precedes or follows a hemorrhage. The smooth ascent of the curve is broken by drops in both free and total acid,

PLATE V
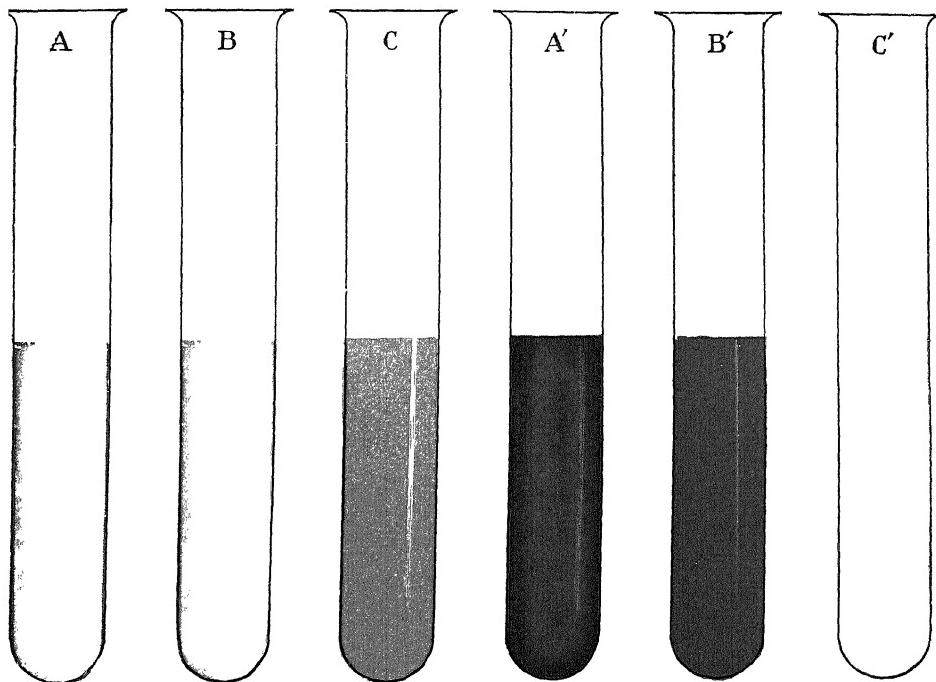
A, gastric fluid to which a 1 per cent solution of phenolphthalein has been added; B, gastric fluid to which a 1 per cent solution of alizarin has been added; C, gastric fluid to which a o 5 per cent solution of dimethylamino-azobenzol has been added; A', A after titration with a decinormal solution of sodium hydroxide; B', B after titration with a decinormal solution of sodium hydroxide; C', C after titration with a decinormal solution of sodium hydroxide (Boston)   (From Todd and Sanford's *Clinical Diagnosis by Laboratory Methods,* W B Saunders Co)

unassociated with biliary regurgitation, followed by a rise to a still higher level than that preceding the drop. The actual acid values are usually much above normal in this type of curve.

4. **Extragastric Curve.**—The acid values, instead of starting to recede after sixty or ninety minutes, continue to rise, the last extraction having the highest acid values. This type of curve is very suggestive of pathology outside of the stomach. Duodenal ulcer, cholecystitis, appendicitis and sigmoiditis are the more common diseases associated with this curve. In duodenal ulcer, appendicitis and early in the course of cholecystitis the acid values are apt to be above normal.

5. **Delayed Digestion Curve.**—Also sometimes called psychical achylia. There is a primary small rise followed by a drop, the acid values remaining very low until sixty or seventy minutes after the meal, when the acid again rises and goes through a normal curve. The primary rise probably represents the psychic secretion and the secondary rise the hormonic. The latter is delayed in appearance.

6. **Hypochlorhydria Curve.**—Hypochlorhydria may be benign or due to carcinoma of the stomach There may be no free hydrochloric acid or but small amounts, being under 40 throughout the two hours. The total acidity is low, being under 40, or reaching about 40 toward the end of the first hour.

HISTAMINE TEST FOR TRUE ACHYLIA

This is conducted in cases of achylia by injecting *subcutaneously* 0.00025 gram (2 5 c.c of 1 : 10,000 solution) of histamine (Hoffmann, La Roche) after the introduction of the tube and the evacuation of the residuum, simultaneously with the ingestion of the meal. It is sometimes conducted with a fasting stomach or after a water meal.

Specimens are removed at fifteen-minute intervals as for fractional analysis, and titrated for total acidity and free hydrochloric acid. If the stomach is capable of producing hydrochloric acid, the glands are stimulated by the reaction produced by the histamine. If acid is not produced, a true achylia is stated to be present.

GASTRIC ANALYSIS BY THE TÖPFER METHOD

Procedure.—1. Feed the Ewald test meal of two pieces (35 grams) of toast and 8 ounces (250 c.c.) of tea.

2. *One hour* later remove the *entire* stomach contents.

3. Make the macroscopic examination as described on page 166 for amount, bile, mucus, pus, odor and gross blood. The amount varies under normal conditions between 50 and 100 c.c. In cases of hypersecretion or defective motility, 200 to 300 c.c. may be found. Very large amounts, as 500 to 3000 c.c , are indicative of dilatation and suggestive of pyloric stenosis.

4. Make chemical analyses for (*a*) total acidity, (*b*) free acidity (free

hydrochloric acid and acid salts), and (c) for free hydrochloric acid with subsequent calculations of (d) combined acidity and (e) acidity due to organic acids and acid salts.

5. In addition, test for lactic acid, occult blood, and protein, and make a microscopic examination.

Determination of Total Acidity.—1. Place 5 c.c. of juice in a porcelain evaporating dish.

2. Add 3 drops of a 1 per cent alcoholic solution of phenolphthalein (see A in Plate V).

3. Titrate with N/10 sodium hydroxide solution until a faint pink color is produced that persists for almost two minutes (see A' in Plate V).

4. Take the buret reading and multiply by 20 to obtain the amount of N/10 sodium hydroxide required for the neutralization of 100 c c. of juice. Normally this is 40 to 60.

5. If it is desired to express the acidity in terms of hydrochloric acid, by weight, multiply the value just obtained by 0.00365.

Determination of Free Acidity (Free Hydrochloric Acid and Acid Salts). —1. Place 5 c.c. of juice in a dish.

2. Add 3 drops of a 1 per cent solution of sodium alizarin sulphonate in water (see B in Plate V).

3. Titrate with N/10 sodium hydroxide until a *distinct violet color* is obtained (see B' in Plate V). It is difficult, without practice, to determine when the right color has been reached. A reddish-violet first appears. The tendency is to add too much sodium hydroxide. The color of the end-reaction can be imitated fairly well by adding 2 or 3 drops of the indicator to 5 c.c. of a 1 per cent solution of sodium carbonate.

4. Take the buret reading and multiply by 20 to determine the free acidity of 100 c.c.

Determination of Free Hydrochloric Acid.—1. Place 5 c.c. of juice in a dish.

2. Add 4 drops of a 0.5 per cent solution of dimethyl-amino-azobenzene in 95 per cent alcohol (see C in Plate V).

3 Titrate with N/10 sodium hydroxide until the initial red color is replaced by orange-yellow (see C' in Plate V). If there is no free hydrochloric acid the juice does not turn pink or red upon the addition of the indicator.

4 Take the buret reading and multiply by 20 to determine the free hydrochloric acid in 100 c.c. The calculations are made as follows:

(a) Total acidity as determined above.

(b) Free hydrochloric acid as determined above.

(c) Subtract the free acidity (alizarin indicator) from the total acidity (a) to obtain the so-called *combined hydrochloric acid* (a protein salt of the acid).

(d) Subtract the free hydrochloric acid (b) from the alizarin acidity (free hydrochloric acid and acid salts) to obtain the *acid salts*.

5. The report is now made as follows:

   (*a*) Total acidity
   (*b*) Free hydrochloric acid
   (*c*) Combined hydrochloric acid (protein salt of hydrochloric acid)
   (*d*) Acid salts

6. If only 3 to 5 c.c. of juice are available, make the titrations with 1 c.c. amounts and use N/100 sodium hydroxide instead of N/10 Multiply by 10 to obtain the amount of N/10 required for 100 c.c. amounts of juice.

7. From the standpoint of accuracy it is recommended to use 10 c.c. amounts of juice for the three titrations.

DETERMINATION OF LACTIC ACID

Kelling's Test.—This is a fairly satisfactory color test depending upon the formation of ferric lactate.

1. Fill a test tube with water.

2. Add 2 drops of a 10 per cent solution of ferric chloride to give a faint canary yellow. Mix well.

3. Pour one-half into a second tube for a control.

4. To one tube add 1 c.c. of strained stomach juice.

5. If lactic acid is present a deep yellow color develops.

Strauss's Test.—This method depends upon extracting the lactic acid with ether and thereby removing such disturbing factors as hydrochloric acid, digestion products, etc. It also gives an approximate idea of the amount.

1. Place 5 c c. of strained gastric juice into a small separatory funnel with a stopper (Fig. 106).

2. Add 20 c.c. of ether and shake thoroughly.

3. After separation of the ether, allow the fluid to run out except the upper 5 c.c. of the former.

4. To the ether extract, add 20 c c. of distilled water and 2 drops of a 10 per cent solution of ferric chloride.

5. Shake gently.

FIG. 106—SEPARATORY FUNNEL.

6. A slightly green color is produced in the presence of 0.05 per cent lactic acid whereas 0.1 per cent yields a very intense yellowish-green color.

Uffelmann's Test.—1. Add 5 c.c. of filtered gastric juice to 50 c.c. of ether and shake thoroughly for ten minutes.

2. Collect the ether and evaporate to dryness.

3. Dissolve the residue in 5 c.c. of water and add to the reagent.

4. If lactic acid is present the solution will develop a canary yellow color.

5. This test may be applied to gastric juice direct without first extracting with ether but this is not as reliable as the above because of interfering substances, such as phosphates, sugars, etc.

Mix 3 drops of concentrated solution of phenol and 3 drops of aqueous solution of ferric chloride. Dilute with water until the mixture assumes an amethyst-blue color.

DETECTION OF OCCULT BLOOD

Benzidine Test (Recommended).—1. Place a knife point full of benzidine in a test tube (use Merck's blood-testing benzidine).

2. Dissolve in 2 c.c. glacial acetic acid. Warm the solution if necessary.

3. Add 2 c.c. of hydrogen peroxide (3 per cent).

4. Add 10 to 15 drops of the fluid to be tested.

5. A positive reaction is indicated by the appearance of a blue or green color.

6. This test is stated to detect blood when present in a dilution of 1 : 3,000,000.

Gregersen's Test (Alvarez and Wight's Modification).—1. Mix a small portion of feces with a few drops of water.

2. Add a few drops of benzidine solution. In the presence of blood a blue color develops.

3. Make the readings with a watch according to the following scale:

$$+ = \text{blue green in thirty to sixty seconds}$$
$$+ + = \text{vivid blue in fifteen seconds}$$
$$+ + + = \text{deep blue in three seconds}$$

4. This test is sensitive in a dilution of blood of 1 :200,000 and does not give reactions with iron salts or products from an ordinary diet.

Barium peroxide...................... 40 grams
Benzidine, pure....................... 5 grams

Mix and store in the dark as stock. Just before use, dissolve about 0.225 gram (this can be estimated) in 5 c.c. of 50 per cent acetic acid.

Other methods are the aloin, guaiac and orthotoluidine tests described in the chapters on examination of urine and feces.

DETECTION OF BILE

Principle.—The following method is based upon the oxidation of the bilirubin with nitric acid to form biliverdin (green).

Procedure.—1. Place about 1 inch of powdered ammonium sulphate in a test tube and add 10 c.c. of gastric juice.

2. Shake vigorously for a minute.

3. Add 3 c.c. of acetone and thoroughly mix by inverting the tube six times (do not shake).

4. Allow the acetone to separate.

5. Allow a drop of nitric acid to flow down the side of the tube.

6. A green color is a positive reaction. If too much acid is used the bili-verdin will be oxidized to a purple or red.

7. If the gastric juice is of a deep green color, dilute 4 or 5 drops with 10 c.c. of water and proceed as above.

PROTEIN ESTIMATION

Principle.—This method depends upon the precipitation of protein by phosphotungstic acid. By using serial dilutions of gastric juice a quantitative result is obtained. Material for this test should be taken from an empty stomach as normally retained foods give positive reactions. While securing stomach contents it is also necessary to avoid contamination with saliva and bronchial secretions as these may yield positive reactions.

Procedure (Wolff-Junghan).—1. Filter gastric juice until clear.

2. Arrange a series of six test tubes in a rack.

3. Place 9 c.c. of distilled water in the first tube and 5 c.c. in each of the remaining five.

4. To the first tube add 1 c.c. of filtered gastric juice and mix thoroughly.

5. Remove 5 c.c. from the first tube and place it in the second tube; mix thoroughly and remove 5 c.c. and place in the third tube, and so on until the sixth tube from which 5 c.c. will be discarded.

6. The tubes will now contain the following dilutions of gastric juice: 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, and 1 : 320.

7. Overlay the contents of each tube with 1 c.c. of Wolff's reagent.

WOLFF'S REAGENT

Phosphotungstic acid...................... 0.3 gm.
Hydrochloric acid (conc.).................. 1.0 c.c.
Alcohol (96 per cent)..................... 20.0 c.c.
Distilled water to make.................. 200.0 c.c.

8. The appearance of a white ring at the point of contact of the fluids constitutes a positive reaction.

9. Note the highest dilution which gives an *immediate positive reaction.*

10. Positive reactions are obtained normally in dilutions up to 1 : 40.

11. In fractional gastric analysis, conduct the test with each sample and chart the results.

12. Under normal conditions the protein concentration follows that of acidity rather closely, but in some diseases of the stomach and notably in carcinoma the protein may be increased out of all proportion to the latter.

DETECTION OF PEPSIN

Principles.—Pepsinogen is normally secreted by the stomach. It has no digestive power until transformed into pepsin by free hydrochloric acid (to a lesser extent by organic acids and the protein salt of hydrochloric acid). Its presence is detected by the digestion of egg albumin.

Qualitative Procedure.—1. Place 25 c.c. of gastric juice in a small flask. If the specimen does not contain free hydrochloric acid, add a few drops of 10 per cent hydrochloric acid.

2. Place in the flask with the gastric juice a disk of coagulated egg albumin. Stopper and place in an incubator at 37° C.

EGG ALBUMIN DISKS

(a) Boil an egg very slowly until the albumin is distinctly coagulated.

(b) Cut the albumin into small cylinders about 5 millimeters in diameter.

(c) Section the cylinders into small disks about 1 millimeter thick.

(d) The disks can be preserved in glycerin until needed, but should be washed in water before using.

3. If pepsin is present the disk will begin to swell in from one-half to one hour and dissolve in about three hours.

Quantitative Procedure (Method of Mett Modified by Nirenstein and Schiff).—1. Introduce into a small Erlenmeyer flask 1 c.c. of gastric juice and 15 c.c. of N/20 hydrochloric acid (0.18 per cent hydrochloric acid).

2. Add two Mett tubes prepared as indicated below, stopper the flask to prevent evaporation and place in an incubator at 37° C. for twenty-four hours.

3. By means of a low-power microscope and a millimeter scale (graduated to half millimeters) determine accurately the length of the column of albumin digested at each end of the tubes. It is well to run the determination in duplicate, in which case the result is the average of the eight figures obtained.

4. Ordinarily from 2 to 4 millimeters of albumin are digested by normal human gastric juice.

5. The peptic power is expressed as the square of the number of millimeters of albumin digested. This is based on the Schütz-Borissow law that the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested. Therefore a gastric juice which digests 2 millimeters of albumin contains four times as much pepsin as one which digests only 1 millimeter of albumin. For example, if the microscopic reading gives on an average 2.2 millimeters of albumin digested, the pepsin value for the diluted juice would be $2.2 \times 2.2 = 4.84$ and for the pure undiluted juice, $4.84 \times 16 = 77.44$.

PREPARATION OF METT TUBES (CHRISTIANSEN'S METHOD).—The liquid portions of the whites of several eggs are mixed and strained through cheese-cloth. The mixture should be homogeneous and free from air bubbles. It is best to allow the egg white to stand for two or three hours in a vacuum

desiccator to remove air more completely. A number of thin-walled glass tubes of 1 to 2 millimeters internal diameter are thoroughly cleaned and dried and cut into lengths of about 10 inches. These are sucked full of the egg white and kept in a horizontal position. Into a large evaporating dish or basin 5 to 10 liters of water are introduced and heated to boiling. The vessel is then removed from the fire and stirred with a thermometer until the temperature sinks to exactly 85° C. The tubes filled with egg white are immediately introduced and left in the water until it has cooled. The tubes thus prepared are soft boiled, more easily digested than hard-boiled tubes, and free from air bubbles. The ends are sealed by dipping in melted paraffin or sealing wax (preferably the latter), and the tubes can be kept thus for a long time. When ready for use, mark with a file and break into pieces about 3 or 4 inches long. After cutting, the tubes should be immediately introduced into the digestion mixture or may be kept a short time under water. Tubes whose ends are not squarely broken off must be rejected.

The digestibility of different egg whites varies widely. Hence, in making up a new set of tubes, if we wish our results to be comparable these tubes must be standardized against those first prepared. This may be done by running simultaneous tests with tubes from the two series, using the same gastric juice and comparing the lengths of the column digested in each case. Christiansen's method of preparing tubes of the same digestibility is to be preferred. He proceeds as in the original preparation of the tubes except that as the water cools from 90° to 80° C. a single tube containing the new egg white is dropped in at each degree change of temperature, that is, at 90°, 89°, etc. Pieces of each of these tubes as well as of the original standard tubes are then allowed to digest simultaneously in portions of the same gastric juice. One of these tubes should show a digestibility equal to that of the standard tubes. For example, the tube coagulated at 88° C. may show the proper digestibility. Then the new series of tubes should be made in the same manner as this one; that is, introduced at 88° C. The tubes thus prepared should be again checked up with the standard to see that no mistake has been made.

DETERMINATION OF TRYPTIC ACTIVITY

Principles.—Trypsin is not secreted by the stomach but occurs in the pancreatic juice. It may be found, however, in the stomach contents because of regurgitation of duodenal contents through the pylorus. *Since it is destroyed by the pepsin-hydrochloric acid of the stomach, the determination must be made immediately after securing gastric juice, especially in cases of high acidity.*

Procedure (Spencer).—1. Prepare five reagent tubes; more if desired.

2. To tubes 1 and 2 add 0.5 c.c. of gastric contents (filter if cloudy).

3. To tubes 2, 3, 4 and 5 add 0.5 c.c. of distilled water.

4. From tube 2 remove 0.5 c.c. of its mixed contents and add to tube 3.

Mix thoroughly and add 0.5 c.c. from tube 3 to tube 4. Repeat for tube 5.

5. This gives dilutions of gastric contents of 1, 1 : 2, 1 : 4, 1 : 8, and 1 : 16.

6. To each tube add 1 drop of phenolphthalein solution (phenolphthalein, 1 gram; 95 per cent alcohol, 100 c.c.); then add drop by drop a 2 per cent sodium carbonate solution until a light pink color is produced.

7. To tubes 1, 2, 3 and 4 add 0.5 c.c. of casein solution. Tube 5 must receive 1 c.c. of casein solution, since it contains 1 c.c. of the diluted gastric contents. For the casein solution, dissolve 0.4 gram of casein in 40 c.c. of N/10 sodium hydroxide. Add 130 c.c. of distilled water, then 30 c.c. of N/10 hydrochloric acid. This leaves the solution alkaline to the extent of 10 c.c. of N/10 sodium hydroxide, minus about 3 c.c. neutralized by the casein.

8. Incubate for five hours at 40° C.

9. Precipitate the undigested casein by dropwise addition of a solution of the following composition: glacial acetii acid, 1 c.c.; 95 per cent alcohol, 50 c.c.; distilled water, 50 c.c. The tubes in which digestion has been complete remain clear; others become turbid.

10. The tryptic values are expressed in terms of dilution. Thus, complete digestion in tube 3 (a dilution of 1 : 4) shows four times the tryptic power of undiluted gastric juice which is taken as a standard as 1; therefore, its tryptic value is 4.

11. Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

DETECTION OF RENNIN

Principle.—Rennin is an enzyme capable of coagulating the protein of milk. Fresh milk is used, therefore, as the reagent.

Lee's Test.—1. Place 5 or 10 c.c. of fresh milk in a test tube.

2. Add 5 drops of gastric juice.

3. Place in the incubator for fifteen to twenty minutes.

4. If rennin is present, coagulation will occur. In this test it is sometimes difficult to tell whether the rennin or the acid in the gastric juice caused the coagulation; however, rennin is practically always present where there is hydrochloric acid in the stomach and the test is only of value in those cases in which there is no hydrochloric acid, to determine the presence or absence of a true achylia.

Riegel's Test.—1. Place 5 c.c. of fresh milk in a test tube.

2. Add 5 c.c. of gastric juice neutralized with N/100 sodium hydroxide (phenolphthalein as indicator).

3. Place in a water bath at 40° C.

4. If rennin is present in normal amount, coagulation will occur in ten to fifteen minutes.

5. Delayed coagulation indicates a less amount.

MICROSCOPIC EXAMINATION

Procedure.—1. Place a small drop of gastric residuum on a slide and cover with a cover glass.

2. Examine with low and high objectives with the light well out.

3. Mix a drop of fluid with a drop of sudan III on a slide and cover with cover glass. Neutral fat globules will be yellow or red.

4. Mix a drop of fluid with a drop of Lugol's solution on a slide and cover with cover glass. Starch granules will be blue or blue black.

5. Make a thin film on a slide, fix with heat and stain with dilute carbol-fuchsin; dry. Examine with oil-immersion objective for Oppler-Boas bacillus (*Lactobacillus boas-oppleri*), staphylococci, yeast, sarcina, etc. Note the organisms present and whether in small or large numbers, singly, in groups or colonies (masses).
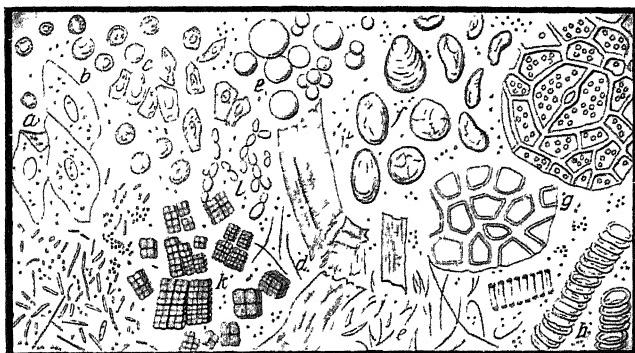
FIG. 107.—MICROSCOPY OF STOMACH CONTENTS

a, squamous epithelial cells from esophagus and mouth; *b,* leukocytes; *c,* cylindric epithelial cells; *d,* muscle-fibers; *e,* fat-droplets and fat-crystals; *f,* starch granules; *g,* chlorophyl-containing vegetable matters; *h,* vegetable spirals; *i,* bacteria; *k,* sarcinae; *l,* yeast cells (Jacob). (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co)

6. With the above preparations examine for the following (Figs. 107 and 108).

Interpretation.—1. *Red blood corpuscles* found in small numbers cannot be considered of any pathological significance. Trauma from passing the tube and aspirating the stomach contents will frequently give rise to their appearance. The consistent finding of large numbers in the absence of considerable trauma may be indicative of ulcer, cancer, erosion, etc.

2. *Leukocytes* are frequently found in the gastric residuum. They are usually partially digested by the gastric juice. They may be indicative of disease from the nose or throat. If so, they are usually associated with other products of inflammation from that region which can easily be identified, *e.g.,* mucus and desquamating epithelium. If these cells are deeply bile-stained and associated with a bilious residuum and other inflammatory elements also bile-

stained (*e g*, columnar epithelium, mucus, bacteria, etc.) it is very suggestive of gall tract disease. The presence of large quantities of pus cells with gastric exfoliative products, mucus and bacteria would suggest gastritis of the infective type. Free pus in the stomach is rare The patient is usually *in extremis* and a rupture of an abscess is usually present, *e g*, subphrenic, pancreatic, biliary, liver empyema, etc. Rarely a suppurative type of gastritis will be the causative agent.

FIG 108—OPPLER-BOAS BACILLI
(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W B Saunders Co)

3. *Mucus* in small amount is practically always found in the gastric residuum. A differentiation between mouth and stomach mucus can often be made by the macroscopic examination. Gastric mucus is usually present as small flocculations which under the microscope look like spherical snail-like bodies. It has a fibrillary appearance and has cellular bodies in its substance. If present in large quantities it is indicative of catarrhal gastritis. Mucus is dissolved by alkalis but not by acetic acid. Mucus from the gall tract should be easy of identification. It occurs in wavy semi-spirals and is rather dense in appearance and stained a deep yellow or greenish yellow if it has resided in the stomach very long. The frequent finding of bile-stained mucus in the fasting stomach is suggestive of gall tract disease. Mucus from the nose is easily recognized by its peculiar tenacity and its staining with pigment or its grayish appearance.

4. *Epithelium* is practically always present in the residuum. The usual cell found is the squamous type. It has no special significance and is derived from the esophagus or above. It is swallowed with mucus during the passage of the tube. Cellular elements from the stomach mucosa are of the columnar variety. They are rarely seen in the normal residuum. Cells are quickly digested in the stomach and unless they are present in considerable numbers they will not be found In atrophic gastritis cellular elements from the gastric tubules are often found. The acid or parietal cells are probably the more easily recognized of the tubule cells. They are about midway in size between a leukocyte and a squamous cell. With eosin and hematoxylin stain the acid cells are stained red and the central or peptic cells blue. The peptic cells take the stain very poorly and are very difficult to recognize, there being nothing but the nuclei remaining. They are much smaller than the acid cells, being a little larger than a leukocyte. They have a long oval nucleus often with just a shred of protoplasm attached. The acid cells, on the other hand, have a very distinct nucleus and the entire protoplasm stains with a fine stippling of the granules The diagnosis of atrophic gastritis may be made upon finding large numbers of acid cells upon repeated examinations. In chronic catarrhal gastritis desquamating elements from the gastric mucosa may be found, even without a severe grade of atrophy being present. The association

of these cellular elements from the gastric mucosa with colonies or groups of pathogenic organisms is indicative of an infective type of gastritis. The frequent presence of deeply bile-stained columnar epithelium in the fasting stomach associated with bile is suggestive of gall tract disease.

5. *Bacteria* in the gastric residuum are frequently of importance. In the ordinary tube examination a few bacteria will usually be found in the normal residuum. However, they will be associated with oral or nasal epithelium or mucus. In other words, they have been swallowed during the passage of the tube. The presence of bacteria, particularly of the pyogenic variety, occurring in colonies or masses and intimately associated with exfoliating epithelium from the stomach, usually means an infective type of gastritis. The presence of masses of deeply bile-stained organisms, on the other hand, may indicate gall tract disease.

The finding of the *Oppler-Boas* bacillus in the stomach is significant of gastric stagnation and nothing else. The Oppler-Boas bacillus when found means cancer in the great majority of cases but to rule out the presence of cancer because of the absence of the organism is folly as the organism will not be found until obstruction has occurred When the Oppler-Boas bacillus is present in cancer the case is far advanced and the diagnosis would be simple without looking for the organisms. In a great majority of the early cases of cancer the Oppler-Boas bacilli will not be found. It is not until the hydrochloric acid starts to be reduced and obstruction has occurred that one can expect to find this organism.

6. *Tissue fragments* are occasionally found in the gastric residuum. Rarely in cases of cancer, small fragments of mucosa may be found showing carcinomatous infiltration and probably more often areas of necrotic tissue containing leukocytes and bacteria. In achylia gastrica and in atrophic gastritis fragments may be recovered showing a great diminution in the number of gastric tubules or a complete absence of tubules. A small round cell infiltration of the fragment may be present in achylia gastrica according to Einhorn. In cases of ulcer small fragments of gastric mucosa may be found. This rarely happens, however, as the hyperacid juice, commonly found in this condition, soon digests any protein matter present. The finding of fragments of mucous membrane showing a hyperplasia of the glandular elements particularly of the base of the gland would suggest a diagnosis of hypertrophic glandular gastritis. Einhorn describes a disease characterized by the appearance of hemorrhagic flakes of gastric mucosa in the fasting residuum which he calls "erosions of the stomach."

7. *Starch granules* are usually present and easily recognized by their concentric striations. When undigested, they stain blue with Lugol's solution; when partially digested, a reddish color due to erythrodextrin. Fat may be present, as likewise other particles of partially digested food such as muscle fibers, vegetable cells, etc. Various crystals may be found, especially of fats, but they possess no particular significance. Animal parasites or ova may be observed.

CHAPTER X

METHODS FOR THE COLLECTION AND EXAMINATION OF BILE AND DUODENAL CONTENTS

COLLECTION

For collection for macroscopic, microscopic and chemical examinations according to the method of Lyon, steps 1, 2, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21 and 22 are recommended. When cultures are to be made for bacteriological examination extra care against contamination is required and the *additional* steps 3, 4, 5, 10 and 18 are recommended.

Procedure.—1. The patient is requested to report in the morning after a fast of twelve to fifteen hours.

2. The night before and likewise before passage of the tube, the patient should brush the teeth and thoroughly rinse and gargle the mouth with an astringent solution.

3. After this the mouth and throat are to be thoroughly rinsed and gargled with a 1 : 500 solution of potassium permanganate (approximately 1 grain to the ounce). This amount of attempted disinfection appears to act better after the tubules and ducts of the buccal membrane have been partly unplugged of their contents.

4. Following this the patient thoroughly rinses and gargles again with the astringent solution, and finally repeats this procedure with sterile water.

5. In cases subject to nasal catarrh, rhinitis or nasopharyngitis, it is a good plan to spray and disinfect the nares as effectually as possible.

6. A duodenal tube, freshly sterilized by boiling and by steam, is swallowed and passed to the greater curvature represented by the first mark on the duodenal tube, which should be placed at 55 centimeters, and which, in the average case, closely approximates the distance between the lips and the greater curvature of the stomach. No water should be swallowed as the tube is being taken, as this would tend to carry contaminating material into the stomach. All patients should be quietly reassured and patiently instructed to take deep, even breaths as the tube is being passed. A method for passing the tube is described on page 182 and shown in Figure 109.

7. The fasting gastric residuum is now extracted by gravity or by syringe aspiration into a conical graduated glass vessel, and notes should be carefully recorded of its amount, color, gross consistency, and other characteristics.

8. The stomach is now washed by repeated douching and withdrawal of 250 c.c. units of sterile water introduced at body temperature from the gradu-

ated 250 c.c. cylinder placed about 18 inches above the patient's head, and continued until the wash water is clear. Attention is paid to and notes recorded of the number of 250 c.c. units required to clean the gastric mucosa of its surface of slime; of the amount and general gross characteristics of the mucus floccules recovered; of the occurrence of biliary regurgitation during this lavage; of the recovery of part or all of each unit given; of a determination of the tonus of the stomach; and the proper functioning of the pyloric sphincter.

9. The gastric mucosa is now astringed by introducing a 250 c.c. unit of
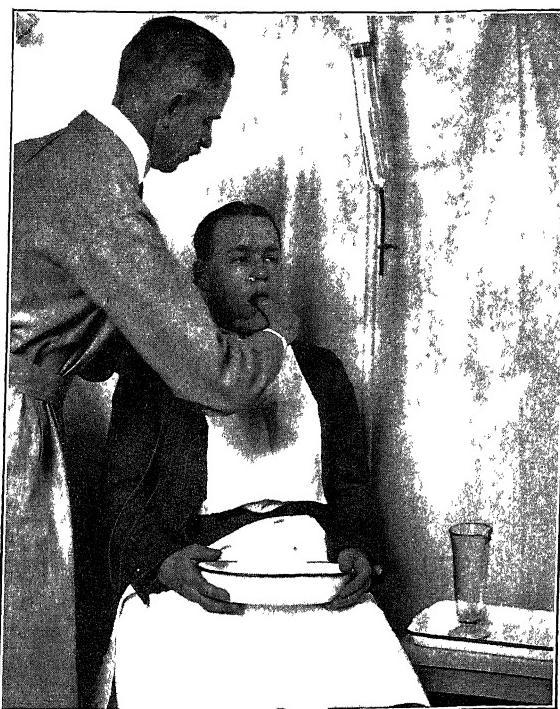
FIG 109—PASSING THE GASTRODUODENAL TUBE (Lyon).
(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger.)

sterile warm water to which has been added 25 c.c. of the astringent solution. This solution is withdrawn and the gastric mucosa is subsequently washed and rewashed until the returned water is again clear. The effect of this astringent is to compress (literally to shrink) the gastric mucosa and, by so doing, press out from the tubular ducts floccules which are to be microscopically examined.

10. An attempt is now made to disinfect the stomach by washing with 250 c.c. of freshly prepared 5 per cent solution of silvol. This is withdrawn and the stomach is again washed and rewashed with sterile water, until the final 250 c.c. is clear and contains no visible flocculent particles.

11. The proximal end of the tube is now clamped off, the patient lies down on a bed or a couch and turns well on the right side, assuming the right Sims position (Fig. 110) and thereafter *very slowly* swallows the tube to the duodenal mark, taking at least twenty minutes *by the watch* to swallow this 20 centimeters of additional tubing. It is fundamentally important to impress upon the patient the necessity of *great care directed against consciously swallowing any saliva* He should be provided with a clean enamel spitting dish or pus pan which he must use for this purpose. The total amount of saliva secreted and spat out during the examination is recorded and in many cases
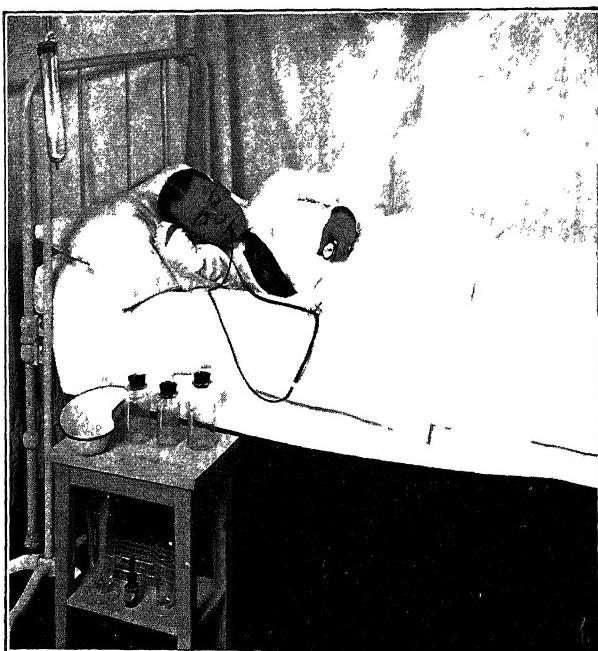
FIG 110—DUODENAL LAVAGE IN SIMS' POSITION.
(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)

should be studied, for certain diagnostic inferences can be secured from attention to this detail.

12. When the tube has been introduced to the second mark (29 inches from tip) by the patient, the position of the tip must be ascertained. The following procedures will be found to be of most value in this connection:

(a) The presence of a "duodenal tug" is suggestive of the position of the tube tip in the duodenum. When the syringe is attached to the tube and an effort made to aspirate, a distinct tug will be felt on the plunger of the syringe, the latter tending to return to the previous position. As suction is made with the syringe and simultaneously with the tug, the tube will often collapse. When the tube is in the stomach, the plunger can usually be withdrawn from

the syringe with very little effort and without feeling a tug. A sharp angulation or kinking of the tube in the stomach, or nestling of the tip apertures against the mucosa of the stomach, will also cause a tug. Needless to say, when the tug is experienced, pulling on the plunger might traumatize the mucosa and cause gross or occult bleeding.

(b) The appearance of golden yellow bile upon aspiration is a point in favor of the tube being in the duodenum. If it is alkaline in reaction and forms a golden yellow froth upon shaking, and the amount is not above 5 to 10 c.c., it is still more indicative of the tube being situated in the duodenum. It must be borne in mind, however, that bile is frequently aspirated from the stomach. Its appearance, however, is quite different from the bile obtained directly from the small intestines. The acid gastric juice causes it to be oxidized and its color becomes green. Bile which has resided in the stomach for a short period quickly becomes turbid. The reaction of the turbid green bile is acid.

(c) The stethoscopic test of Lyon is very satisfactory. Connect a one-ounce syringe to tube and inject air in quick spurts, and at the same time listen with a stethoscope placed over the exposed epigastrium. If the sound is heard over a considerable area with the point of maximum intensity to the left of the midline, and approximately as much water or air can be withdrawn as was injected, the tip of the tube is now probably in the stomach. If the sound is localized to the right hypochondrium, but no peristaltic bubbling or crepitant sounds are set in vibration and no air can be withdrawn, the tip is probably in the grip of the antrum pylori, but has not passed through. If the sound is sharply localized in its maximum intensity to the right hypochondrium, and peristaltic crepitant sounds are set up by the injection of air, and only small fractions of air can be withdrawn, the tip is probably in the duodenum.

If the tube has not entered the duodenum it must be pulled out as far as the stomach mark and again slowly reswallowed.

(d) The water test is very reliable. The patient is given a half glass of water to drink. If the water can be immediately aspirated, the tube tip is in the stomach. The failure to obtain water through the tube after it has been swallowed is a point in favor of its being in the duodenum. This method should not be used when a culture from the bile is going to be made as it may wash bacteria from the mouth and throat.

If there is still doubt after the above methods have been tried, the following procedure used by Einhorn may be tried: One ounce of warm water is allowed to run into the tube by gravity. Before the water completely disappears from the syringe, pinch the tube and allow it to syphon out. The warm water will frequently relax the sphincter of Oddi sufficiently for a small amount of bile to run into the duodenum and the water will return distinctly bile-tinged and alkaline in reaction.

The only method which is absolutely reliable is the fluoroscopic examina-

tion for direct visualization of the tube. Frequently, however, this is not available and considerable experience with the above methods will make the fluoroscope only occasionally necessary.

13. Occasionally, difficulty may be experienced in getting the tip of the tube through the pylorus. In this event, 4 ounces of sterile water are introduced *through* the tube to supply the stomach with a fluid menstruum and encourage gastric peristalsis. The water must not be swallowed by the patient because esophageal débris would thereby be washed into the stomach.

This difficulty is encountered in but a minority of patients but when it is, the following conditions must be considered:

(*a*) *Faulty technic:* (1) Tubes coming from the manufacturer may be marked incorrectly. Every new tube should be measured and marked properly before being used. (2) Too rapid swallowing of the tube is a frequent cause of its failure to pass into the duodenum.

(*b*) *Pylorospasm:* This is a not infrequent cause of failure. If the tube has been reswallowed twice without success or there is reason to suspect pylorospasm, introduce through the tube 1 ounce of water containing 15 to 20 drops of tincture of belladonna; atropine sulphate subcutaneously and intragastrically has no advantage over belladonna. Benzyl benzoate has not proven of any great value in this connection. If still unsuccessful after belladonna, and pylorospasm is suspected as the cause, the patient is given tincture of belladonna in physiologic dosage for three or four days and asked to report for a second attempt at bile drainage.

(*c*) *Gastroptosis and gastric atony:* Difficulty is often experienced in intubating the duodenum in this type. The right lateral position, almost on the stomach, with the hips raised off the table by a pillow, is often of service during the swallowing of the tube. This causes the stomach to fall over to the right and away from the spine and helps to straighten out the sharp antral and duodenal angulation which is present in the fishhook stomach. Allow the patient to swallow several additional inches of tube rather than pull the tube out to the 21-inch mark, if it has not entered the bowel in twenty minutes. Success will usually follow the modification of the usual technic in these cases, but some patients of this type have always caused difficulty even after repeated attempts. They are exceptions, however, and a large proportion of people of enteroptotic habitus can be intubated without difficulty.

(*d*) *Organic disease of the stomach and duodenum·* The first hint of organic disease is sometimes derived from failure to intubate the duodenum. If, associated with the inability to get the tube through the pylorus, gross or occult blood is obtained upon aspirating, a suspicion of organic disease should be entertained and roentgen-ray study should always be made. Organic obstruction of the stomach or duodenum, due to peptic ulcer or carcinoma, is the usual offender. Peptic ulcer near the pylorus, not causing organic obstruction, may be accompanied by persistent pylorospasm which simulates actual organic obstruction because of its constant presence. Gastric ulcer, far re-

moved from the pylorus, may cause persistent pylorospasm, but this is unusual. Another cause of organic obstruction encountered is that due to perigastric or periduodenal adhesions, more often the result of gallbladder disease. If the obstruction is due to adhesions, a position can often be found which will allow the tube to enter the duodenum.

14. Having determined the position of the tip to be in the duodenum, a little air is injected to balloon out the duodenal walls and thus avoid trauma when gentle suction is exerted on the syringe. The duodenal fluid is drained out and laid aside for microscopical examination, pancreatic ferment determination and occult blood test. If bile-stained material is obtained before stimulation with magnesium sulphate, it is obvious that the common duct sphincter is open and part or all of the *A,* or common duct, bile has escaped, and cannot be differentially figured on.

15. The gallbladder is stimulated to evacuation of its fluid contents by instilling into the duodenum 75 c c. of 33 per cent volumetric solution of magnesium sulphate at body temperature. This percentage is actually equivalent to a 16 66 per cent solution of magnesium sulphate, or 25 c.c of a saturated solution plus 50 c.c. of water. This solution is run in by gravity through the barrel of the syringe. The end of the tube is pinched to hold its syphonage, and then attached to a drainage bottle. Suction by bulb or syringe is not applied unless fluid does not run out under gravity syphonage (under a pressure head of 18 to 24 inches), and then only by a 1-inch bulb.

16. When bile begins to flow past the "window" in the tube, the collection bottle is changed and subsequent alterations in the gross appearance of the bile are followed by changing the collection bottle. The interpretation of the first bile obtained depends upon biliary regurgitation observed during step 10 and the condition of Oddi's sphincter as determined under step 13. If bile has been obtained at either of these two points the first bile obtained after stimulation with magnesium sulphate is probably *C* bile.

17. If the bile stops flowing or no *B* bile is obtained, the patient is restimulated one or more times, depending upon the amount of magnesium sulphate solution that has been retained.

18. Cultures are made as desired from any bile sample. For gallbladder cultures the best material is afforded by the last of the *B* bile (the dregs from the floor of the gallbladder) This is theoretically so but is practically difficult of accomplishment. Cultures are taken directly into glucose hormone bouillon in the special "fool-proof" flasks devised by Richardson (Fig. 111). Or the bile may be collected in a sterile vial (Fig. 112) and plated in the laboratory.

19. When no further bile is being obtained or the drainage session is to be stopped, a fresh solution of silvol 1 : 5000 (using 10 c.c. of a 1 : 500 solution added to 90 c.c. of sterile water) is instilled into the duodenum and immediately withdrawn by syphonage. Amounts up to 75 per cent of this are usually obtained, but only rarely is the entire amount recovered.

20. The patient now sits up and is given a duodenal enema consisting of

250 c.c. of Ringer's solution at body temperature. This should be run in slowly by the drip method in not less than twenty minutes. This may be reenforced by adding from one-quarter to one teaspoonful of the crystals of sodium sulphate as may be necessary to secure two to four fluid intestinal evacuations within three hours following the drainage. The amount of sodium sulphate to be added to the Ringer's solution will depend upon the amount of the magnesium sulphate previously used and retained by the patient, and upon the average response of the individual patient to saline cathartics.

21. On completing the duodenal enema a little air is injected to free the

FIG III —SPECIAL CULTURE FLASK
(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger.)

metal tip by ballooning out the duodenal wall, and the tube gently but evenly withdrawn. Momentary obstruction may be encountered at the pylorus, at the cardia and at the glottis. At the first two points, or elsewhere, except at the glottis, injecting a little air will release the obstruction. The retention of the tip of the tube at the glottis is promptly overcome by the patient swallowing simultaneously as the tip is withdrawn.

22. The patient is given a pleasant mouth wash to rinse the mouth, and is then given a cup of beef broth and a few crackers. He is permitted to go home and may often go about his business, but is encouraged to rest as

quietly as possible. The total duration of this treatment averages two and a half to three hours. Where liver drainage is an essential in treatment the tube is left in and drainage continued for a longer period up to about six hours.

Kinds of Duodenal Fluid and Bile.—1. The fluid obtained when the tube reaches the duomenum is duodenal juice, which may contain some bile and gastric juice. It may be labeled *"D"* and is generally 10 c.c. or less and rarely over 20 c.c.

2. When the magnesium sulphate solution has been introduced and removed, it is replaced by a light golden-yellow bile, which Lyon believes is principally derived from the common bile duct with some from the cystic and hepatic ducts with perhaps a few

FIG. 112—COLLECTION VIAL.

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)

drops from the gallbladder. It is labeled *"A"* bile (see Fig. 1 in Plate VI) and normally averages from 5 to not more than 30 c.c.

3. If drainage is continued the bile deepens to a darker golden-yellow to yellow-brown with a viscosity between that of syrup and thin molasses. Lyon believes that this is derived from the gallbladder and is labeled *"B"* bile (see Fig. 2 in Plate VI) ; it averages 30 to 45 c.c.

4. Under normal conditions this is followed by very much lighter and thinner lemon- to straw-colored bile which Lyon believes is freshly secreted and expelled liver bile designated as *"C"* (see Fig. 3 in Plate VI). It may drain for a long time, even up to several hours, and amounts to 30 c.c. or more.

5. The addition of gastric juice to any of these fractions may convert them to a greenish color (probably oxidation with production of biliverdin) with turbidity and sometimes effervescence due to the action of hydrochloric acid upon the salts.

MACROSCOPIC EXAMINATION

Under pathological conditions the amount, color and physical characteristics of *A, B* and *C* biles may undergo marked changes. A record and report should cover the following:

1. Amounts of each.
2. Color.
3. Clear or turbid (turbidity may be due to access of gastric juice and is not necessarily indicative of disease).

4   Presence or absence of white or bile-stained flakes   The amount of these in *uncentrifuged* specimens is of considerable importance   A very occasional floccule is normal   Large numbers are indicative of inflammation and may be recorded as follows

$$+ = \text{few (normal)}$$
$$++, +++, ++++ = \text{pathological}$$

5   Precipitates resulting from admixture of gastric juice with bile must not be mistaken for flakes

6   Viscosity (normal or increased by mucus)

7   Blood (pure blood rarely observed)

MICROSCOPIC EXAMINATION

1   Flakes or sediment may be secured after standing or by centrifuging, placed on slides, covered with glasses and examined microscopically
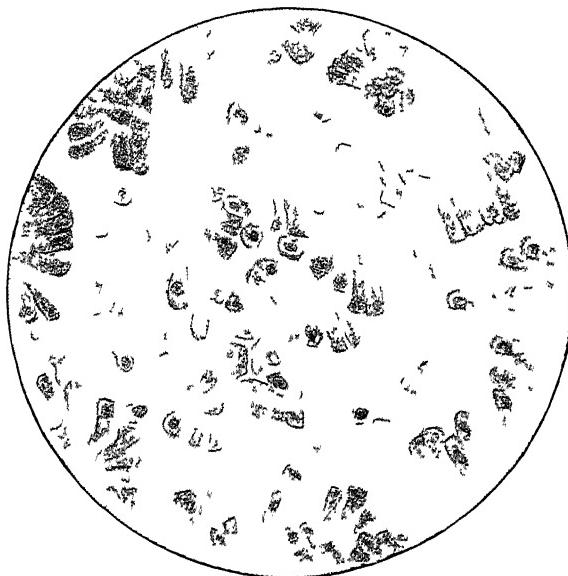
FIG 113 —SHORT COLUMNAR BILE-STAINED EPITHELIUM
(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)

2   Selected material may be likewise smeared on slides, dried in the air, stained (Gram's method or by methylene blue or dilute carbolfuchsin) and examined microscopically

3   As the cells (epithelium and leukocytes) may undergo degenerative changes upon standing it is advisable to make the microscopic   examinations immediately or within an hour after the collection of specimens

4.   The microscopy of bile is capable of yielding very important information

PLATE VI
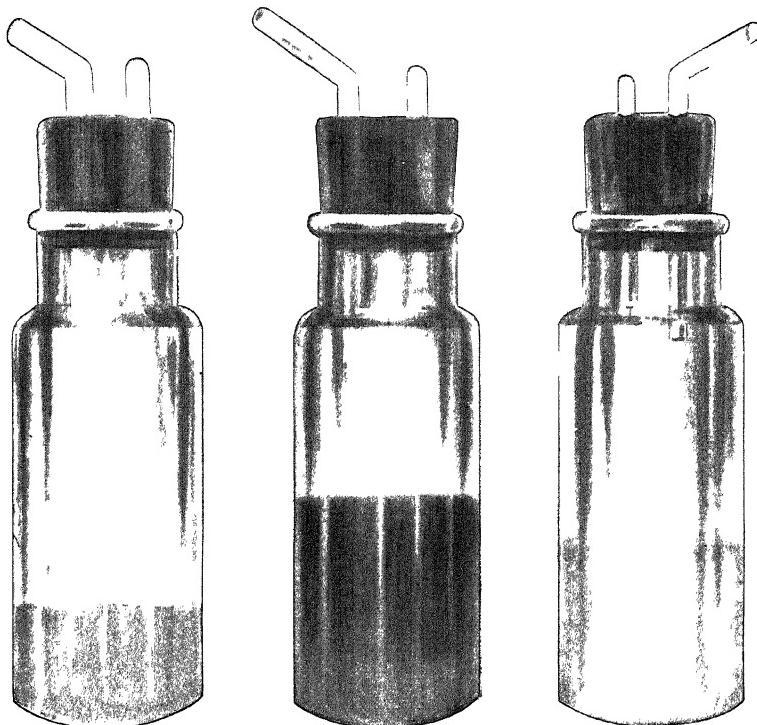
NORMAL A, B AND C FRACTIONS OF BILE

FIG 1 —Common duct   FIG 2 —Gall bladder   FIG 3 —Liver bile
bile (chiefly)   bile (chiefly)   (pure)

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)

but requires considerable experience for reliable results, especially in the differentiation of the kinds of cells.
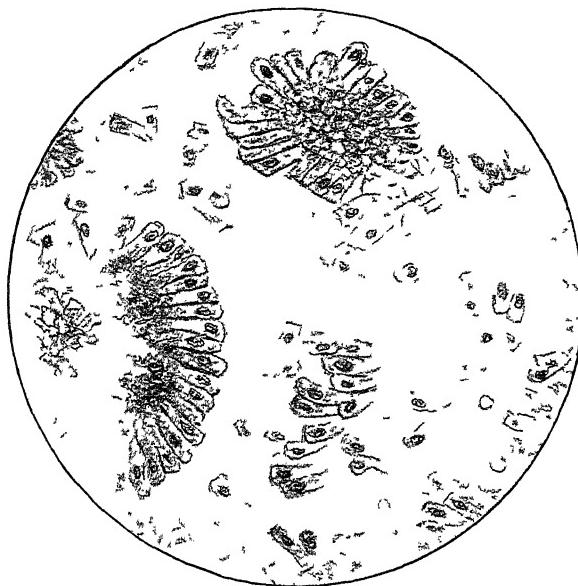
FIG 114—TALL COLUMNAR BILE-STAINED EPITHELIUM

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)
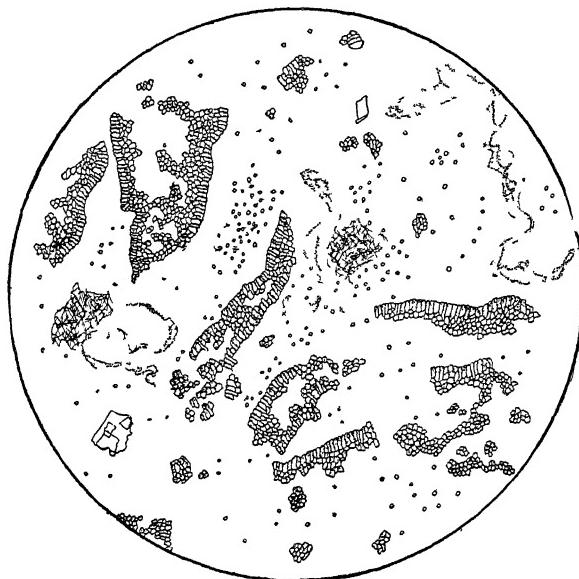
FIG 115—DEGENERATED BILE-STAINED COLUMNAR EPITHELIUM

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)

5. *Normally* a centrifuged specimen will show a few desquamated epithelial cells, an occasional leukocyte, an occasional strand of mucus and sometimes a few crystals and bacteria.

6. *Epithelial cells* may be oval, cuboidal, tall or short columnar, etc, and may occur singly or in clusters, rosettes, etc   They may show varying stages of degeneration.   *It is important to note whether or not they are bile-stained*
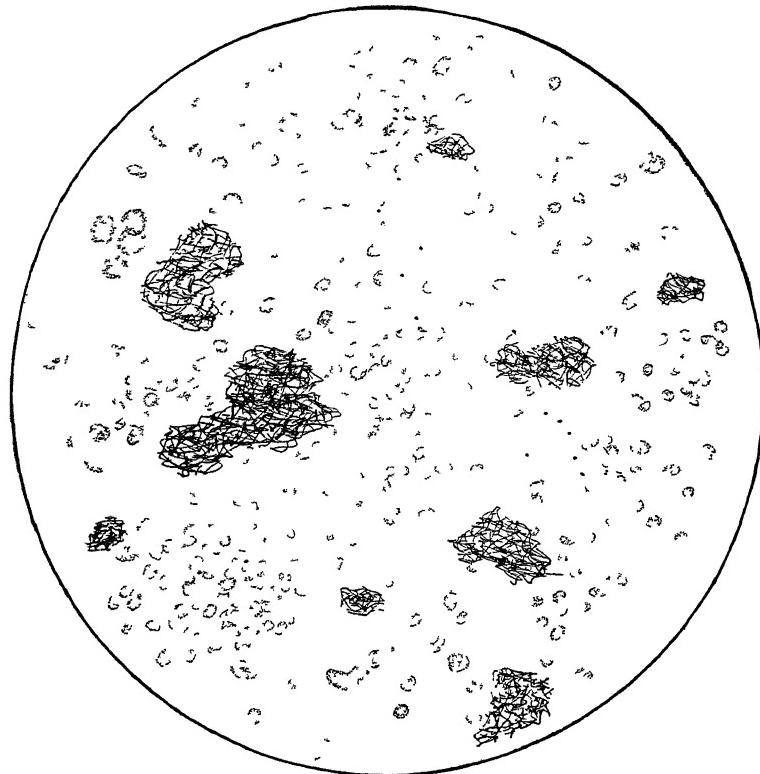
FIG 116—PRACTICALLY PURE PUS WITH OCCASIONAL NECROTIC EPITHELIAL CELL AND MASSES OF BILE PIGMENT

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)

(*a*) Oval, cuboidal or low columnar cells (not bile-stained) are probably from the duodenal mucosa.

(*b*) Columnar cells stained with bile may come from any part of the biliary tract.

(*c*) Short bile-stained columnar cells are probably from the smaller gall ducts (Fig. 113).

(*d*) Tall columnar bile-stained cells are probably derived from the gall-bladder and larger gall ducts. When very deeply stained they are usually from the gallbladder (Fig. 114).

(*e*) Upon standing for two hours or more, epithelial cells may undergo degenerative changes with loss of nuclei and granular degeneration of cytoplasm, producing so-called "shadow cells" (Fig 115).

7. Note average number of *leukocytes* per field and whether or not stained with bile (Fig 116).

8. *Mucus* strands usually occur in long, waxy and curled strands and may be bile-stained

9. *Crystals* are of considerable diagnostic importance:

> (*a*) Cholesterin: colorless, thin, rectangular or square crystals (most common) (Fig 117) They are usually indicative of gallstones
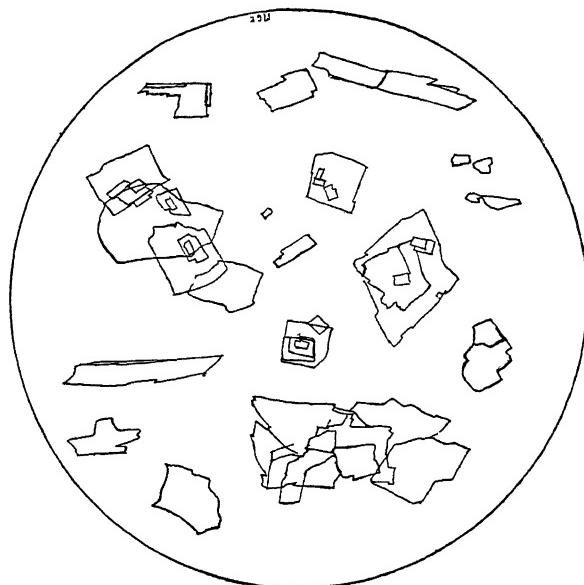
FIG 117—CHOLESTERIN CRYSTALS

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger)

> (*b*) Leucin: round, thick, yellowish, concentric crystals (rare)
>
> (*c*) Tyrosin· long, slender needles (rare)
>
> (*d*) Calcium salts: (rare). Calcium bilirubin crystals found in association with cholesterol are practically pathognomonic of cholelithiasis

10 *Pigment* (so-called bilirubin calcium pigment) occurs as a lustrous, golden-orange substance in irregular masses frequently composing small sand particles It is more frequently encountered than cholesterin in cases of cholelithiasis

11. *Bacteria* are of significance when present in large numbers as clumps

and irregular masses (Fig. 118). They are usually gram-negative bacilli of the colon group, staphylococci and streptococci, but identification requires cultures.

12. *Precipitates,* largely due to mixture of gastric juice with bile with a precipitation of bile salts, present a granular appearance devoid of cells.

13. The following *parasites* or ova may be found, especially in the duodenal juice (D): (a) *Uncinaria americana,* and (b) *Lamblia intestinalis* (*giardiasis*), especially enmeshed in strands of mucus.
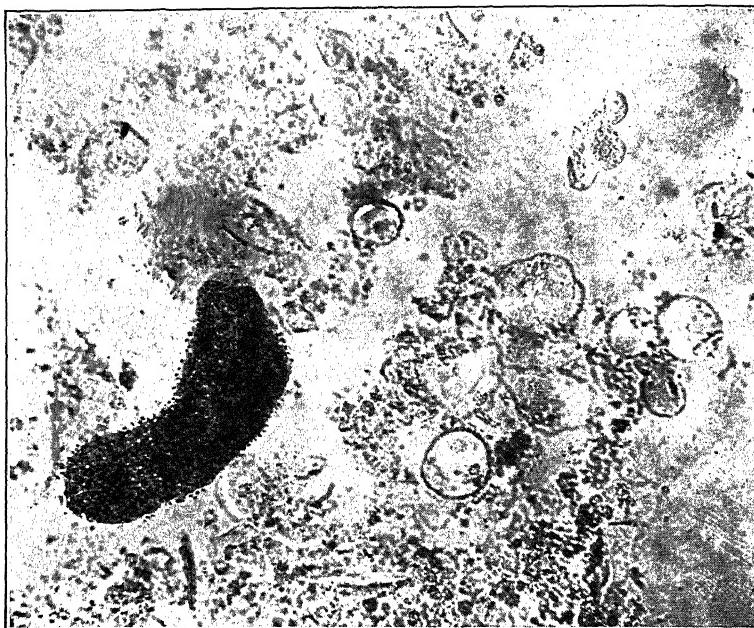
FIG. 118.—BACTERIAL COLONY FROM DUODENUM.

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger.)

DETECTION OF TRYPSIN, AMYLASE AND LIPASE IN DUODENAL CONTENTS

Einhorn's Method.—1. Place sufficient bile in test tube to make about 3 inches deep.

2. Place in the bile the following tubes (file off the ends, avoiding dried out ends):

    (a) Hemoglobin tube
    (b) Starch tube
    (c) Olive oil tube

PREPARATION OF HEMOGLOBIN TUBES

Hemoglobin powder...................... 1.0 gm.
Agar powder............................ 2.5 gm.
Water (distilled) q.s. .... 100.0 c.c.

Rub hemoglobin powder with 10 c.c. of water until a smooth paste is obtained; then add the agar and water. Heat and fill capillary tubes as directed for starch tubes.

PREPARATION OF STARCH TUBES

Agar powder............................ 2.5 gm.
Starch................................. 5.0 gm.
Water (distilled)....................... 100.0 c.c.

Rub starch and agar in a mortar with sufficient water to make smooth paste, then add balance of the water.

Place in a flask and heat to boiling point.

Draw by suction while still hot into warm capillary glass tubing (inside diameter 1.5 millimeters).

After cooling cut into small pieces (3 centimeter lengths) and seal the ends with paraffin.

PREPARATION OF OLIVE OIL TUBES

Olive oil.............................. 25 c.c.
Agar powder........................... 2 gm.
Nile blue sulphate (aq. sol. 1 : 2000) q.s..... 100 c.c.

Rub olive oil and agar together to make thin paste and then add Nile blue solution to make 100 c.c. Heat to boiling; fill in capillary tubes as directed for starch tubes.

3. Place in the incubator at 37° C. for twenty-four hours, and examine as follows:

4. Examine the two ends of the hemoglobin tube for clear, digested area; measure each with millimeter rule; take the average and report in numbers of millimeters. The digestion is due to the presence of trypsin.

5. Examination of starch tube. Push the contents of tube out on a slide and cover with Lugol's solution; measure the part which does not turn blue as above and report in millimeters. The digested portion fails to give the starch reaction with iodine. The digestion is due to the presence of amylase.

6. Examine the ends of the olive oil tube and measure the portion which has turned violet. The Nile blue in the digested portion turns violet. Report in number of millimeters as above. The digestion is due to the presence of lipase.

BACTERIOLOGICAL EXAMINATION

1. Smears of unstained and stained material may be examined for preliminary data as described above.

2. Cultures should not be made if there is excessive retching and gagging because of the chances of contamination.

3. Cultures are indicated (a) when large numbers of organisms are seen in smears; (b) in cases requiring a careful search for foci of infection; (c) in cases of obvious gallbladder infection in which vaccine therapy is to be tried.

4. The technic is described in Chapter XVIII and the bacteriological findings in Chapter XIX.

CHAPTER XI

METHODS FOR CONDUCTING LIVER FUNCTIONAL TESTS

VAN DEN BERGH TEST FOR BILIRUBIN IN BLOOD

Principle.—When a color develops in a blood serum on adding diazo reagent, the time of appearance and deepening of the color are taken as indicating the type of jaundice present. By precipitating the proteins with alcohol and saturated ammonium sulphate after adding the diazo reagent, the amount of bilirubin present is determined by comparison with a standard.

Reagents.—SOLUTION A.—Dissolve 1 gram sulphanilic acid in 15 c.c. concentrated hydrochloric acid in water and dilute to 1000 c.c.

SOLUTION B.—Dissolve 0.5 gram sodium nitrite in water and dilute to 100 c.c.

DIAZO REAGENT.—To a 10 c.c. graduated cylinder add 5 c.c. of solution A and 3 drops of solution B. Prepare fresh before each determination.

SATURATED AMMONIUM SULPHATE.—In a bottle place 100 grams ammonium sulphate and 100 c.c. water. Shake for some time to dissolve and use the supernatant fluid.

STANDARD COBALT SOLUTION.—Weigh out 2.161 grams of anhydrous cobalt sulphate or 3.92 grams of $CoSO_4 + 7H_2O$, dissolve in water and dilute to 100 c.c. in a volumetric flask.

STANDARD SERIES.—To seven centrifuge tubes of 15 c.c. size add respectively 10, 7.5, 5, 4, 3, 2, and 1 c.c. of the standard cobalt sulphate solution. To the last six add the correct amount of water to make 10 c.c. Stopper and label respectively 2, 1.5, 1, 0.8, 0.6, 0.4, and 0.2 milligrams. The standard cobalt sulphate solution is equivalent to 0.5 milligram bilirubin or 1 Van den Bergh unit. The standard tubes are labeled to read directly in milligrams of bilirubin considering the 1 : 4 dilution in the method.

Procedure.—DIRECT REACTION.—1. Blood is drawn into a plain tube and allowed to clot, centrifuged, and the serum removed. There must be no hemolysis. A dry syringe and needle should be used. This test may be done on oxalated blood. The serum must be clear and free of hemoglobin, especially for the direct reactions.

2. Pipet 1 c.c. serum in a small test tube, add 2 c.c. water, and 0.5 c.c. of the diazo reagent. Mix. The reaction may be one of the following:

(*a*) *Negative.* Most sera with a normal bilirubin content do not give the direct reaction.

(*b*) *Positive.* (1) Immediate direct: The color change to red begins within thirty seconds and reaches its maximum within a minute or so. (2) Delayed direct: The color change begins after thirty seconds and gradually deepens. This may require some minutes. (3) Biphasic direct: A combination of two preceding reactions.

INDIRECT REACTION.—1. Pipet into a 15 c.c. centrifuge tube 1 c.c. of the serum and add 0.5 c.c. of the diazo reagent.

2. Let stand two to three minutes.

3. Add 2.5 c.c. of 95 per cent alcohol and 1 c.c. of saturated ammonium sulphate.

4. Mix and centrifuge at a relatively high speed for fifteen minutes.

5. Compare the supernatant liquid with the series of standards. In case it is above 2 milligrams, remove carefully 1 or 2 c.c. of the supernatant liquid and dilute with 50 per cent alcohol until a match can be made with one of the standards. Multiply this reading by the number of times the fluid has been diluted. The standards and the unknown are often not quite the same shade. Hemolysis will give a brown tinge to the unknown. The reading on the standard tubes is milligrams bilirubin per 100 c.c. blood.

NOTES.—1. Normal serum contains 0.1 to 0.3 milligram bilirubin. The immediate direct reaction is said to indicate obstructive jaundice.

2. The delayed direct reaction is said to indicate jaundice of the hemolytic type.

3. It is essential that *all tubes and pipets be perfectly clean.* The color of the standard and unknown are not identical as a rule. When 2 c.c. of serum are available it is more convenient to run the determination using double amounts of reagents. If the unknown is too strong for accurate comparison, dilute with a solution composed of 2 parts 95 per cent alcohol and 1 part distilled water.

4. The diazo reagent is added before alcohol to allow "coupling" to take place. By this method very little if any bilirubin is carried down with the precipitated protein, as the azobilirubin is very soluble in alcohol while bilirubin is less so and is carried down with the precipitate in relatively large amounts if the reagents are added in reverse order.

5. Chylous sera give cloudy solutions which are objectionable for colorimeter comparisons.

6. Values obtained with specimens preserved in the ice box are probably correct; values obtained with specimens standing for twenty-four hours at room temperature are not reliable.

7. Slight hemolysis does not interfere.

8. Plasma may be used instead of serum.

DETERMINING THE ICTERUS INDEX

Principle.—The intensity of color of bilirubin of blood serum is compared with a standard potassium dichromate solution.

Reagents.—SODIUM CHLORIDE.—Dissolve 9 grams sodium chloride in water and dilute to 1000 c.c.

POTASSIUM DICHROMATE STANDARDS.—Dissolve 1 gram potassium dichromate in water, add 2 drops concentrated sulphuric acid, and dilute to 100 c.c. in a volumetric flask. Into eleven dry, clean, ordinary test tubes pipet 10, 5, 3, 2, 1.5, 1.2, 1, 0.7, 0.5, 0.3, and 0.1 c.c. of the above dichromate solution, and with a 10 c.c. Mohr pipet dilute the last ten tubes to 10 c.c. with distilled water. Mix. Clean eleven small test tubes (10×100 millimeters) with cleaning solution, wash and dry. Fill about two-thirds full with the above solution. Label the tubes respectively 100, 50, 30, 20, 15, 12, 10, 7, 5, 3, 1. These may be tightly stoppered or preferably closed by sealing the glass. These labeled numbers correspond to icteric index units.

Procedure.—1. With a dry needle and syringe, or one washed thoroughly with sterile saline, draw 4 to 5 c.c. of blood into a plain tube and allow to clot. Centrifuge and remove the serum. *There must be no hemolysis.*

2. Place 2 c.c. of the serum into one of the clean, dry, small test tubes.

3. Place in the comparator block and compare with the nearest standard tubes. Read directly as icterus index. In case the color is too deep the serum is diluted with 0.9 sodium chloride and the reading obtained multiplied by the dilution factor.

NOTES.—1. Normal serum has an index of 3 to 5.

2. An index of 6 to 15 is the range of latent jaundice (without clinical signs).

3. Fasting blood should be used as the lipemia after a meal interferes with the reading.

4. Low values may be found in secondary anemia.

5. Yellow pigments in the food (carrots) give a false reading and are to be avoided twenty-four to forty-eight hours before the test.

THE BROMSULPHTHALEIN TEST

Principle.—Bromosulphthalein (phenoltetrabromphthalein sodium sulphonate) when injected into the blood stream is rapidly removed by hepatic cells and excreted into the bile.

Clinical Procedure.—The patient is weighed and the dosage calculated on a basis of 2 milligrams per kilogram of body weight. The body weight of the patient in pounds divided by 55 will give the exact quantity in c.c. of the 5 per cent solution required.

Bromsulphthalein is prepared by Hynson, Westcott and Dunning in sterile ampules containing a 5 per cent solution. The dye may be measured by draw-

ing it into a sterile 5 c.c. syringe. It is then slowly injected directly into an arm vein. The injection should be sufficiently slow to occupy one minute, and care should be taken not to allow infiltration outside the vein. Thirty minutes after injection, a sample of blood (4 or 5 c.c.) is drawn, preferably from the opposite arm, by allowing the blood to run into a dry test tube. In cases of early liver disease it may be advisable to obtain a sample of blood at exactly five minutes after injection.

Standards and Reagents.—The standards may be prepared by adding 40 milligrams (0.8 c.c. of 5 per cent solution) of bromsulphthalein to 1000 c.c. of water alkalinized with 2.5 c.c. of a 10 per cent solution of sodium hydroxide. This represents the 100 per cent standard. By proper dilutions with similarly alkalinized water, ten standards are prepared, ranging from 10 to 100 per cent. Five c.c. of each standard may be sealed in small test tubes and no deterioration in color will occur for several months if they are kept in the dark.

Permanent standards with comparator box may be obtained from Hynson, Westcott and Dunning.

SODIUM HYDROXIDE.—Approximately 10 per cent.

HYDROCHLORIC ACID.—Approximately 5 per cent by volume.

Procedure (Rosenthal and White).—1. Centrifuge and separate serum soon after the specimen reaches the laboratory.

2. Divide serum, putting half in each of two test tubes.

3. To one tube add 1 drop of 10 per cent sodium hydroxide.

4. To the other add 1 drop of 5 per cent hydrochloric acid.

5. Estimate the amount of dye present by comparing with a series of standards. The tube of clear acidified serum is placed behind the standard in a suitable comparator box and a tube of water is placed behind the unknown.

NOTES.—1. Thirty minutes after injection the serum of normal individuals is entirely free from the dye or contains only a faint trace. Five minutes after injection from 20 to 50 per cent of the dye (average 35 per cent) is present in the serum.

2. The percentage of dye in the serum may be interpreted directly in terms of percentage of impaired liver function.

CHAPTER XII

METHODS FOR THE EXAMINATION OF FECES

MACROSCOPIC EXAMINATION

In the complete routine examination of feces, attention should be given the following·

1. *Quantity* The average adult stool varies from 100 to 200 grams (3 to 10 ounces) but is usually around 100 grams. It is much larger (up to 350 grams) upon a vegetable diet.

2 *Form and consistency.* (*a*) Hard and scybalous as in constipation; (*b*) soft and formed (normal); (*c*) mushy or liquid, as in diarrhea; (*d*) gaseous (fermentative); (*e*) flattened or ribbon-like, as in spastic colitis or obstruction, etc.

3 *Color* is greatly influenced by diet and drugs:

  (*a*) Light or dark brown (normal) due to hydrobilirubin
  (*b*) Yellow (milk diet; rhubarb; senna; santonin; unchanged bilirubin)
  (*c*) Green (spinach and other chlorophyllic vegetables; calomel; biliverdin)
  (*d*) Clay (deficiency of bile in jaundice)
  (*e*) "Acholic" (undigested fat; jaundice)
  (*f*) Dark red or chocolate brown (excess cocoa or chocolate)
  (*g*) Black (iron; bismuth suboxide; charcoal, blood)
  (*h*) Red (undigested blood; beets)

The *"separation test"* for intestinal motility is conducted by giving just before one of the meals of the day a gelatin capsule (No. oo) containing 0.2 to o 3 of a gram of carmine or charcoal (former preferred). An inspection is then made of the subsequent stools and a note made of the time elapsing between the ingestion of the capsule and the appearance of a red (carmine) or black (charcoal) stool; normally this is about twenty-four hours.

4 *Odor*, which is normally due to indole and skatol, is greatly influenced by diet and disease. The normal odor is subject to wide variations but can usually be reported under the following designations: (*a*) normal; (*b*) slight; (*c*) almost odorless; (*d*) sour; (*e*) pungent; (*f*) putrid; (*g*) very offensive, etc Odor is very marked in a meat diet, much less so from a vegetable diet and frequently hardly detectable in milk diet.

5 *Mucus* is very important and should always be reported under the fol-

lowing designations: (a) no excess (normal); (b) slight excess; (c) great excess; (d) almost pure mucus. It may be mixed with blood, as in dysentery; appear as firm bands suggesting tapeworms; occur as brown or black jelly-like masses, or be so intimately mixed with the stool as to be detected only upon mixture with water.

6. *Concretions* are usually to be looked for while washing the stool through a sieve with water. Gallstones are faceted and, if doubtful, should be examined for cholesterin and bile pigments. Artefacts resembling stones may be composed of soap and fats following the ingestion of olive oil, etc. Intestinal concretions (enteroliths) are rare. "Intestinal sand" is usually mucus and occurs as sandlike granules. Fruit seeds (pears, apples, grapes, oranges) are sometimes mistaken for concretions until washed and closely examined.
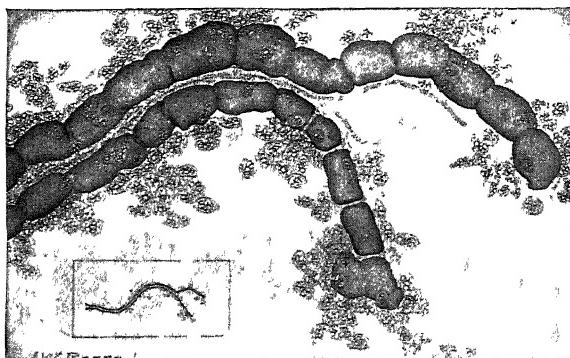
FIG 119—UNDIGESTED FIBER FROM THE CENTER OF A BANANA RESEMBLING A PARASITE
    (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W B Saunders Co)

7. *Parasites* may be readily detected by inspection, but a careful examination requires washing the stool through a sieve. Various artefacts like vegetable tissue from poorly chewed celery or "greens," banana, fruit skins, etc., may lead to errors by hasty, incomplete examination (Fig. 119). Segments of tapeworms, round worms, pin or seat worms are usually easily detected if due care is exercised. The larvae of insects may be found in exceptional instances.

8. *Foods and curds* may be readily seen or detected after washing through a sieve. Record as slight, moderate or large numbers or amounts The curds should be described as large or small; tough or soft, etc.

DETERMINATION OF THE REACTION

Normally the feces are slightly alkaline when freshly passed; usually they are neutral to litmus paper. An acid reaction is much less frequent and when it does occur commonly follows a vegetable diet. Pronounced dietary changes produce at most but minor changes. Infants' stools are generally acid.

1. Examine as soon as possible after defecation.

2. Thoroughly mix the stool and test with red and blue litmus paper. If the stool is hard, mix with water.

3. Test with Congo red paper.

4. To a watery suspension add a few drops of a 1 per cent alcoholic solution of phenolphthalein (turns pink if alkaline).

DETECTION OF BLOOD

Principles.—1. Blood may be detected by macroscopic inspection (streaks; small clots; "tarry") or by microscopic examination.

2. "Occult" or hidden blood may be detected by any of the following chemical methods.

3. As only small amounts of material can be examined and as traces of blood may be unevenly distributed, it is recommended that the specimen be well mixed before taking portions for the tests described. Furthermore it is always advisable to apply at least two tests if any one of them gives a positive reaction.

Benzidine Test.—1. In a test tube dissolve a knife-tip of benzidine (special for blood tests) in 2 c.c. of glacial acetic acid, and add 20 drops of a 3 per cent solution of hydrogen peroxide.

2. Make a smear of the feces on a slide and pour the reagent over it (Wagner).

3. It turns blue if blood is present (almost instantaneously in the presence of large amounts).

4. Or make a thin suspension of feces in about 5 c.c. of water and heat to boiling to inactivate the oxidizing enzymes.

5. To the reagent (prepared as above) add 2 or 3 drops of the cooled fecal suspension.

6. A clear blue color indicates a positive reaction.

Phenolphthalein Test.—1. Make a thin suspension of stool in about 5 c.c. of distilled water and heat to the boiling point to inactivate the oxidizing enzymes.

2. After cooling, add 2 c.c. to 1 c.c. of the reagent and add a few drops of hydrogen peroxide.

3. A pink or red color is a positive reaction.

4. The reagent is prepared by dissolving 2 grams of phenolphthalein and 25 grams of potassium hydroxide in 100 c.c. of distilled water. Add 1 gram of powdered zinc and heat gently until the solution is decolorized. Does not deteriorate on standing.

Orthotoluidine Test (Ruttan and Hardisty).—1. To 1 c.c. of a watery suspension of feces in a test tube add 1 c.c. of reagent (4 per cent solution of orthotoluidine in glacial acetic acid) and 1 c.c. of 3 per cent hydrogen peroxide.

2. In the presence of blood a bluish color slowly develops.

Guaiac Test.—1. Prepare 5 to 10 c.c. of a watery suspension of feces and place in a test tube.

2. Add an equal volume of ether and shake vigorously.

3. Allow ether to separate and discard.

4. Add one-third volume of glacial acetic acid and mix.

5. Add ether; shake well; allow to separate.

6. Place about 5 c.c. of the ether extract in a test tube and add a few granules of gum guaiac.

7. Mix well and add a few c.c. of hydrogen peroxide.

8. A violet or blue color is a positive reaction.

DETECTION OF UROBILIN

Principles.—1. The word "urobilin" is here used as a synonym for hydrobilirubin and to include its mother substances, bilirubin and the chromogen, urobilinogen.

2. Owing to constipation and other factors, the amount is subject to variation, although the total daily output is fairly uniform.

3. Since the mother substance, bilirubin, is a product of hemoglobin, an estimation of urobilin in the feces is an approximate index of blood destruction and has had a useful application in differentiating between pernicious anemia due to blood destruction and the secondary anemias due to hemorrhage.

4. Urobilin is absent or greatly reduced in obstructive jaundice and its return to the feces is often the first sign of relief.

Schmidt's Qualitative Test.—1. This test depends upon the production of hydrobilirubin-mercury with the production of red color.

2. Rub up a small amount of feces in a mortar with a saturated watery solution of mercuric chloride.

3. Transfer to a shallow white dish and let stand for six to twenty-four hours.

4. The presence of hydrobilirubin or urobilin is indicated by a deep red color being imparted to the particles of feces containing the pigment.

5. If unaltered bilirubin is present, a green color is produced through its oxidation to biliverdin.

Quantitative Test of Wilbur and Addis.—This method depends upon extraction of hydrobilirubin and its quantitative estimation by spectroscopic examination.

1. Collect all the feces for twenty-four hours, keeping them in darkness.

2. Grind the whole quantity with water to a homogeneous paste.

3. Dilute to 1000 c.c. with tap water (or to 500 or 2000 c.c. if the amount of feces is unusually small or large).

4. Measure off 25 c.c. and add to this 75 c.c. acid alcohol (alcohol 64 c.c., concentrated hydrochloric acid 1 c.c., water 32 c.c.).

5. Place in a mechanical shaker for one half hour. Constant shaking by hand for a similar period will suffice.

6. Add 100 c.c. of saturated alcoholic solution of zinc acetate, and filter.

7. To 20 c.c. of the filtrate add 2 c.c. of Ehrlich's reagent (paradimethyl-aminobenzaldehyde, 20 grams; concentrated hydrochloric acid, 150 c.c.; water, 150 c.c.).

8. Keep in darkness until next day (or at least for six hours) and examine spectroscopically. In the presence of both urobilinogen and urobilin, the absorption bands indicated in Figure 120, between b and F, will be seen.
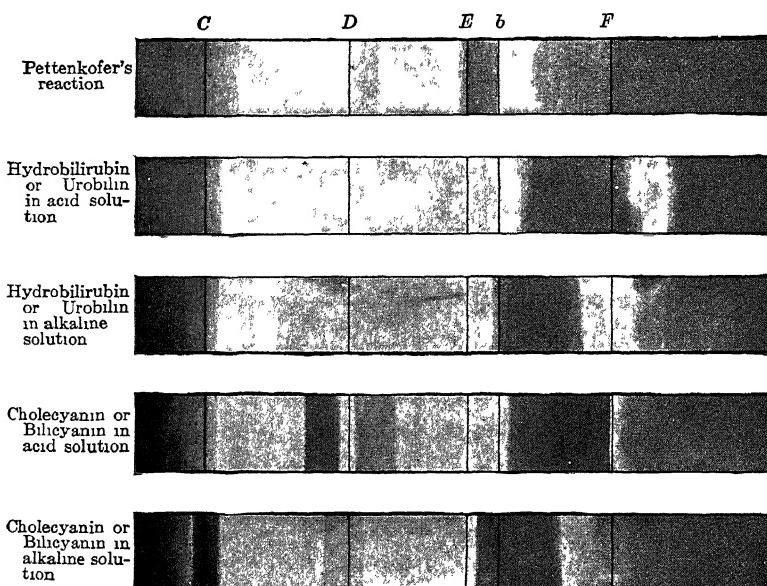
FIG. 120.—ABSORPTION SPECTRA. (Wood.)

9. Dilute with 60 per cent alcohol, adding a few c.c. at a time, until first one and then the other band has entirely disappeared, when the slit of the spectroscope is wide open, but still remains visible when the slit is partly closed. The end-point is fairly definite after one has established his standard upon a series of normal stools. It is perhaps best to use an unvarying width of slit and to dilute until the bands have just disappeared with this opening. One may establish uniform conditions as to the thickness of the layer of fluid, the kind and strength of the light, and the distance from the light, and then adopt a width of slit which gives an average of about 6000 dilutions in a series of normals. When using the "pocket" type of spectroscope, place the fluid in a standard serologic tube about 12 millimeters in diameter, and employ a 60-watt frosted Mazda lamp, placed about 6 inches from the spectroscope, which is mounted upon a temporary stand to insure steadiness. The eyes are protected from the light by a cardboard screen

10. Calculate separately the number of dilutions necessary to cause disappearance of each of the absorption bands and add the two together. The calculation is based, not upon the 20 c.c. of filtrate used, but *upon the 2 5 c.c. of fecal suspension represented by the filtrate*. The dilution value for the twenty-four-hour stool (1000 c.c. of fecal suspension) is then found by multiplying this figure by 400. When the fecal suspension is made up to 500 or 2000 c.c. the multiplier would, of course, be 200 or 800. This final result indicates the number of dilutions which would be necessary if all the urobilin and urobilinogen of the twenty-four-hour stool were concentrated in the 2.5 c.c. of fecal suspension examined.

EXAMPLE: Suppose that in step 9 the urobilinogen band disappeared when the 20 c.c. of filtrate had been diluted to 25 c.c., and the urobilin band when the volume reached 30 c.c., then the dilution values for the 2.5 c.c. of feces would be 10 and 12 respectively and the combined value $10+12=22$. The total dilution value of the twenty-four-hour stool would then be $22\times400=8800$.

DETECTION OF BILIRUBIN

1. Place a few drops of concentrated nitric acid in an evaporating dish or upon filter paper.

2. Apply a few drops of a suspension of feces.

3. The usual play of colors is produced (green, blue, violet, red and yellow). This test may be done on a slide and observed under a microscope.

DETECTION OF BILE ACIDS

1. Extract a small amount of feces with alcohol, and filter.

2. Evaporate the filtrate in a dish over a water bath.

3. Dissolve the residue in water made slightly alkaline with potassium hydroxide solution.

4. Add 5 drops of a 5 per cent solution of sucrose.

5. Transfer to a test tube and carefully run down the sides about 3 c.c. of concentrated sulphuric acid. Cool the tube in running water so that the temperature does not go above 70° C.

6. A red ring at the point of contact is a positive reaction. Upon slight agitation the contents of the tube assume a reddish color.

DETECTION OF PANCREATIC FERMENTS

Principles.—1. Two of the pancreatic ferments—amylase and trypsin—are normally present in feces. Lipase cannot usually be detected.

2. In pancreatic disease and obstruction of the pancreatic duct these ferments may be diminished or absent. Quantitative tests, therefore, may be of diagnostic value, especially in conjunction with an estimation of amylase in the urine.

3. Tests for both ferments should be done although that for amylase is

the more useful of the two, since the action of trypsin may be simulated by erepsin and proteolytic bacteria. Amylase is also secreted by the glands of the small intestines.

Securing Specimen of Feces.—1. Upon the evening before the test, limit the patient to a light supper and give a high enema at bedtime.

2. At 7 next morning give 750 c.c. (25 ounces) of milk.

3. At 7:30 give one half ounce Epsom salts; repeat at 8.

4. At 8:30 give a glass of water containing one quarter teaspoonful of sodium bicarbonate.

5. Save all the feces passed up to 2 P.M. in a vessel containing 2 ounces of toluol. Keep in a cool place. If less than 400 c.c. are obtained, give an enema of 1 pint of water.

6. Dilute the whole volume of feces to 3000 c.c. with normal salt solution, mix well, and centrifugalize a portion for five minutes. Use the supernatant fluid for the following tests.

Tests for Amylase.—1. Prepare a 1 per cent solution of soluble starch as follows: To 100 c.c. of cold distilled water, add 1 gram soluble starch (Kahlbaum's recommended) and heat gently with constant stirring until clear.

2. Place 2 c.c. of this solution in each of twelve test tubes.

3. To these tubes add the supernatant fluid from the centrifugalized feces as follows:

No. 1: 1.8 c.c.	No. 7: 0.6 c.c.
No. 2: 1.6 c.c.	No. 8: 0.4 c.c.
No. 3: 1.4 c.c.	No. 9: 0.2 c.c.
No. 4: 1.2 c.c.	No. 10: 0.1 c.c.
No. 5: 1.0 c.c.	No. 11: 0.05 c.c.
No. 6: 0.8 c.c.	No. 12: none (control)

Bring the quantity in each tube up to 4 c.c. with normal salt solution.

4. Place the tubes in an incubator or water bath at about 38° C. for one half hour.

5. Fill all tubes with tap water and add a drop of weak iodine solution to each. Gram's iodine solution will answer.

6. If amylase be present, the series of tubes will vary from yellow through reddish-purple to pure blue, depending upon complete or partial digestion of the starch. The tube before the one in which the first definite trace of blue appears is taken as the measure of digestion. In normal individuals it is usually found to be either the ninth or tenth tube, corresponding to 30,000 and 60,000 units respectively.

Test for Trypsin.—The well-known Gross test may be applied as follows:

1. Prepare a 1:1000 solution of casein as follows:

Casein (C.P.)	0.1 gm.
Sodium bicarbonate	0.1 gm.
Water (distilled)	100 c.c.

Boil for one minute, stirring constantly, and cool.

2. Place 5 c.c. of the casein solution in each of twelve test tubes and add to these tubes the same amounts of the fecal suspension, previously filtered, as were used for the amylase test.

3. Place the tubes in the incubator or a water bath at 38° C. for one hour.

4. Test for digestion of casein by adding a few drops of 3 per cent acetic acid to each tube and mixing gently. Digestion is complete in those tubes in which no white precipitate forms, and the tube before the one in which the first definite precipitate appears is taken as the measure of proteolytic activity (nearly always the fourth tube). The end-point is less definite than in the test for amylase.

DETECTION OF FATS

Fats occur in neutral fats, fatty acids and soaps, which may be differentiated as follows:

Test	Neutral Fats	Fatty Acids	Soaps
Microscopic appearance	Round or irregular globules; highly refractile or minute needles	Sheaves of large needles or short delicate curved needles which occur in such thick masses that the shape of the individual crystals can seldom be made out	Needles arranged in clusters or fans or in short plump crystals or scales. In amorphous form as gnarled bodies everted like the pinna of an ear. Soap crystals are comparatively coarse, as a rule (thick short needles or flakes), but may be indistinguishable from those of fatty acids
Heat	Melted	Melted	Not melted
Ether solubility	Dissolved	Dissolved	Not dissolved
Scharlach r*....... .	Stained	Crystals not stained. Globules stained	Not stained
Sudan III*	Stained	Light orange crystals not stained	
Water	o	o	Sodium and potassium soaps dissolved. Calcium and magnesium soaps not dissolved

* Scharlach r and sudan III solutions are saturated solutions in equal parts 70 per cent alcohol and acetone

Acetic Acid Test.—By rubbing up a small portion of the feces in about 36 per cent acetic acid, applying a cover slip and heating over a flame until the preparation shows bubbles, soaps and neutral fats can be converted into free fatty acids which show as more or less numerous highly refractile bodies and assume crystalline structure as the preparation cools. This procedure is often not successful.

Quantitative Determination of Fats (Saxon).—The soaps are converted into free fatty acids by means of hydrochloric acid, and the material is then

extracted by shaking with ether. The ether removes the neutral fat, the fatty acids present as such, the fatty acids from the soaps, and the cholesterol. The ether is removed, the crude fat purified by means of petroleum ether, and the weight of the total fat obtained. The fat is then dissolved in benzene and titrated with N/10 sodium alcoholate solution, using phenolphthalein as an indicator. The fatty acid is calculated, from the titration, to stearic acid.

1. Place about 5 grams (accurately weighed) of thoroughly mixed feces in a 100 c.c. glass-stoppered graduated cylinder, care being taken not to smear the neck of the cylinder. This procedure is best carried out by weighing a small evaporating dish of feces along with a small spatula before and after transfer.

2. Add 20 c.c. of distilled water, 1 to 2.5 c.c. of concentrated hydrochloric acid (depending upon the amount of the sample) and sufficient water to make a total bulk of 30 c.c. Add exactly 20 c.c. of ether, stopper, and shake vigorously for five minutes. Allow to stand a few seconds, remove the stopper, add exactly 20 c.c. of 95 per cent alcohol, and again shake for five minutes.

3. Stand the cylinder aside. The ether, containing practically all of the fat, will come to the top as a colored transparent layer. Blow the ether off into a 200 c.c. Erlenmeyer flask, making use of the syphon principle as employed in the wash bottle, the submerged end of the delivery tube being bent upward. The thin layer of ether which remains is diluted with 5 c.c. of ether, the tube slightly agitated, and the ether blown off. This is done in all five times, care being taken each time to wash down the sides of the cylinder. The stopper should also be washed.

4. Twenty c.c. of ether are again added and the cylinder shaken for five minutes and set aside. When the ether has nearly stratified, blow it off and wash as before. During the second washing stratification will complete itself.

5. Using a hot water bath, distill off the ether until no trace of alcohol remains. This is assured by finally bringing the water bath to boil for a few minutes. To the residue add 30 c.c. of low-boiling petroleum ether. Stopper with a cork and allow to stand overnight. (Petroleum ether for this work should boil below 60° C. It should be tested for a residue on evaporation and must be redistilled if such is present.)

6. Filter the petroleum ether solution of the fat, catch the filtrate and petroleum ether washings in a tall 100 c.c. beaker which has been previously heated in an oven at 100° C., transferred to a desiccator and weighed. Evaporate off the solvent on a clean surfaced electric hot plate (being careful not to overheat near the end). Dry in an oven at 100° C., cool in a desiccator for twenty minutes and weigh. The difference in the two beaker weighings represents total fat in 5 grams of feces.

7. After weighing, dissolve the contents of the beaker in 50 c.c. of benzol, heat almost to the boiling point, add 2 drops of a 0.5 per cent solution of phenolphthalein, and titrate with an N/10 solution of sodium alcoholate.

8. The weight of fatty acids (in terms of milligrams of stearic acid) is

obtained by multiplying the number of c.c. of N/10 sodium alcoholate solution by the factor 28.4.

9. The difference between the weight of the total fat and the weight of the fatty acids is the weight of the neutral fat.

NOTE.—In order to facilitate separation of the ether and water it may be desirable to put the cylinder in a centrifuge.

GENERAL MICROSCOPIC EXAMINATION

1. Prepare a thick suspension by rubbing up a portion about the size of a walnut in water (see Figs. 121, 122). This gives a uniform mixture more representative than selecting small bits at random.

2. Place a drop on a slide and cover with a large cover glass (No. 1) for general examination.
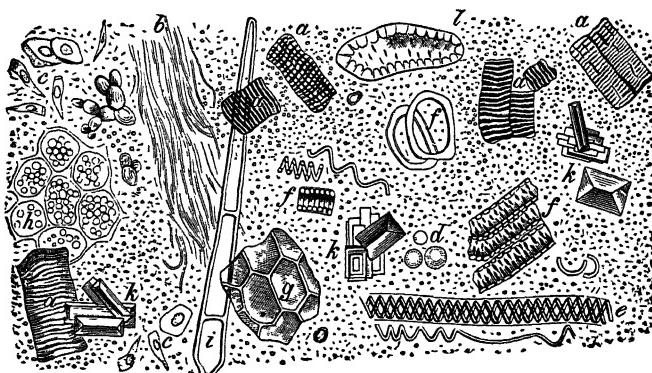
FIG. 121.—GENERAL MICROSCOPIC EXAMINATION OF FECES

(a) Muscle fibers; (b) connective tissue; (c) epithelial cells; (d) leukocytes; (e) spiral vessels of plants; (f, g, h) vegetable cells; (i) plant hairs; (k) triple phosphate crystals; (l) stone cells (after von Jaksch). (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)

3. Place a drop on a slide with 1 or 2 drops of 30 per cent acetic acid (No. 2) for muscle, leukocytes and pus.

4. Place a drop on a slide with 1 or 2 drops of sudan III (No. 3) for fats.

5. Place a drop on a slide with 1 or 2 of Lugol's solution (No. 4) for starches.

6. Examine each microscopically with low and high power with the light well cut down as in the examination of urinary sediments.

7. In this general examination the following may be looked for (see Fig. 121):

(a) Vegetable fibers and hairs.

(b) Connective tissue consisting of colorless or yellowish threads which swell and become gelatinous in the acetic acid preparation.

(c) Muscle. If striations are visible, digestion is imperfect. If the nuclei

are visible, pancreatic function is absent or deficient (see Schmidt's nuclei test).

(*d*) Elastic tissue which generally accompanies connective tissue; outlines more definite with branching; more distinct in the acetic acid preparation.

(*e*) Starch. If undigested the granules are blue on the slide treated with Lugol's solution; reddish if partially digested.

(*f*) Neutral fats. Stain red with sudan III solution; also globules of fatty acids.

(*g*) Leukocytes and pus, which are best seen in the acetic acid preparation. A few are normal; an excess occurs in dysentery and in other inflammatory states masses of pure pus may be seen. In bacillary dysentery Haughwont and others have described *macrophages* consisting of large mononuclear phagocytic cells with large vesicular nuclei, frequently containing remnants of ingested
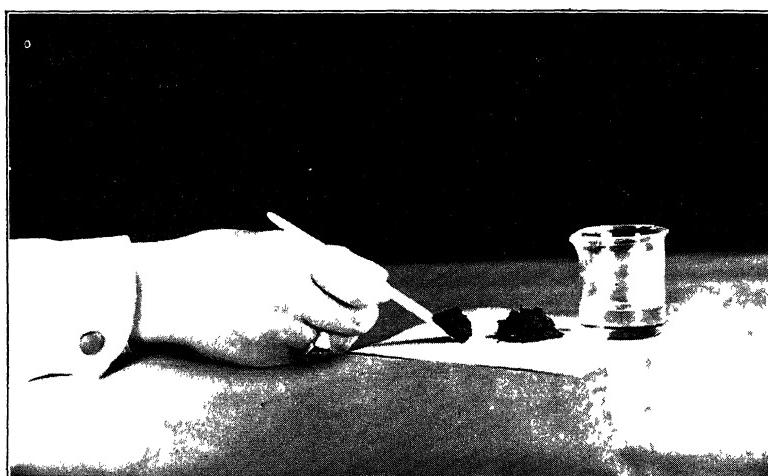
FIG. 122.—TAKING A SAMPLE OF FECES (Benbrook).

leukocytes and erythrocytes. They show varying degrees of necrosis and may present only circular or oval rims with granular débris ("ghost cells"). These macrophages may be mistaken for amebae but can be differentiated by lack of motility and by the character of the nuclei.

An excess of *eosinophils* may be found in the mucus in the discharges of intestinal allergy.

(*h*) Mucus, especially in mucous colitis. dysentery and other diseased states.

(*i*) Erythrocytes, which are best seen in the untreated slide.

(*j*) Epithelial cells, which show all stages of disintegration and are often unrecognizable. A marked excess of recognizable cells may occur in diseased states.

(*k*) Crystals, which ordinarily have but little significance: (1) slender,

needle-like crystals of fatty acids and soap; (2) triple phosphates; (3) calcium oxalate from vegetables; (4) Charcot-Leyden crystals, especially in parasitic infestments; (5) yellowish or brown needles or rhombic crystals of hematoidin after intestinal hemorrhages, etc.

Schmidt's Test Diet.—For a special microscopical examination of the digestion of muscle, starches, and fats, this test diet is recommended.

1. Bowels to be evacuated in the morning and stool discarded.

2. Breakfast of 500 c.c. (about 16 ounces) of milk and 50 grams (about 2 ounces) of toast. Also a gelatin capsule (No. 00) containing 0 2 gram of carmine as a "marker."

3. Forenoon: 0.5 liter porridge, made as follows: 40 grams oatmeal, 10 grams butter, 200 c.c. milk, 300 c.c. water, 1 egg, and salt to taste.

Midday: 125 grams hamburger steak, with 20 grams butter, fried so that the interior is quite rare; 250 grams potato, made by cooking 190 grams potato with 100 c c. milk and 10 grams butter, the whole boiled down to 250 c.c.

Afternoon: Same as morning.

Evening: Same as forenoon.

4. Examine stools as soon as carmine appears as described above.

Schmidt's Nuclei Test.—This test is especially designed for the study of pancreatic function and consists of: (1) The administration of a 0 5 centimeter cube of beef or, better, of thymus, tied in a little gauze bag with the test meal. The meat must have been hardened previously in alcohol and well washed in water. (2) When the bag appears in the feces it is opened and its contents examined microscopically by pressing out small bits between a slide and cover. A drop of some nuclear stain may be applied if desired. (3) If the nuclei are for the most part undigested, pancreatic insufficiency may be assumed, since it is probable that nuclei can be digested only by the pancreatic juice. (4) Normally the nuclei are digested, provided the time of passage through the intestine is not less than eight or ten hours. Upon the other hand, if the time of passage exceeds thirty hours, nuclei may be partially digested in the complete absence of pancreatic juice.

MICROSCOPIC EXAMINATION FOR OVA AND PARASITES

Shearer's Method (Benbrook Modification).—1. Pick up at least 1 gram of feces, using tongue blade, and place it in sufficient water to liquefy it. Do not use too much water (Fig. 122).

2. Thoroughly mix the feces with the water (Fig. 123).

3. Coarse particles may be removed if necessary by straining (Fig. 124).

4. Fill a test tube or centrifuge tube nearly half full of the fecal mixture (Fig. 125).

5. Add to the above an equal quantity of sugar solution prepared as follows:

<div style="text-align:center">

Granulated sugar............................ 1 lb.

Water..................................... 12 oz.

</div>

Dissolve the sugar in the water, by immersing the bottle in hot water. Add 1 per cent phenol as a preservative.
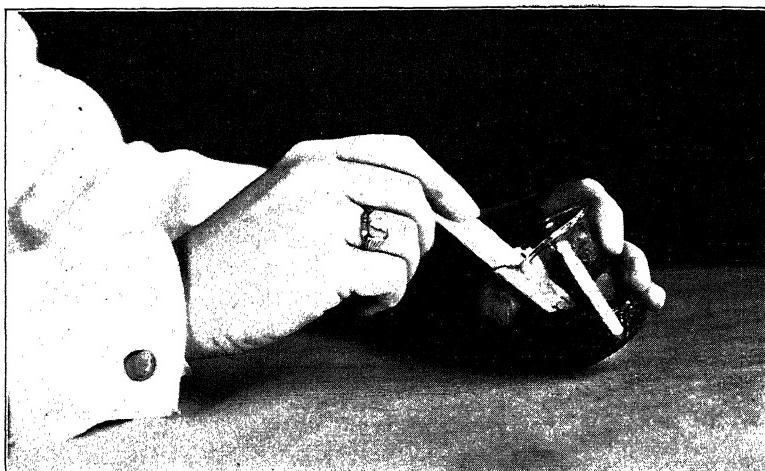
FIG. 123.—MIXING FECES WITH WATER (Benbrook).

6. Mix by slowly inverting the tube several times.

7. Centrifuge the tube containing the mixture for about three minutes at moderate speed, 1500 to 2000 revolutions per minute. Centrifuging may be omitted if the tube is allowed to stand twelve to twenty-four hours.

8. Remove the tube from the centrifuge to a test tube holder without shaking.

9. Lift off the surface layer of fluid (which now contains the eggs) from the tube by means of a headed glass rod prepared as follows: Heat one end of a 6 inch length of 5 millimeter glass rod until it is soft enough to be "headed" against a cold metal object. The head portion should be just slightly less in diameter than the inside of the tubes used. A heavy glass rod, slightly smaller than the inside of the tube, may be used in place of the headed rod. The rod should be slowly lowered into the tube and the instant full contact is made with the liquid withdraw the rod quickly, bringing with it a large drop.

10. Transfer the drop from the rod to a microscope slide by gently rotating the rod in the center of the slide (Fig. 126). A second or third drop may be added to the first in order to obtain sufficient material to fill in under a micro cover slip.

11. Carefully lower a micro cover slip on the drop without pressure.

12. Examine the slide under the low power of the microscope. For best
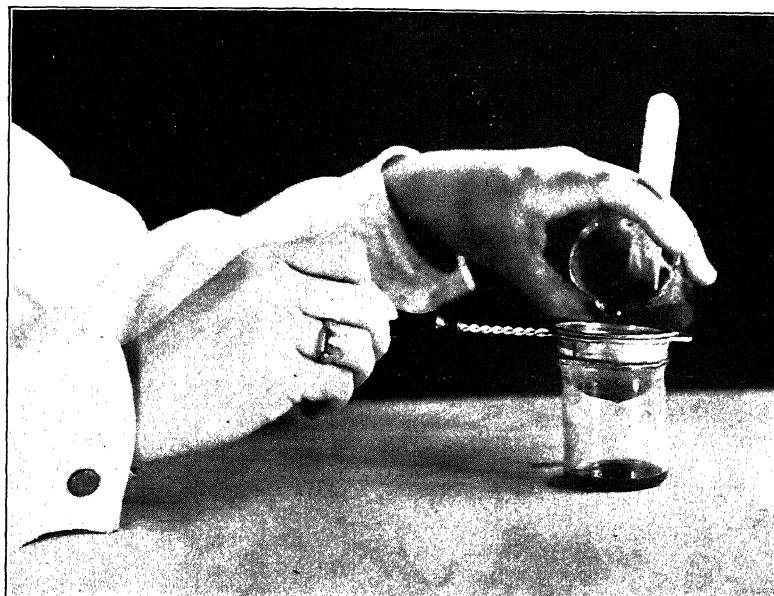
FIG. 124.—STRAINING FECES (Benbrook).
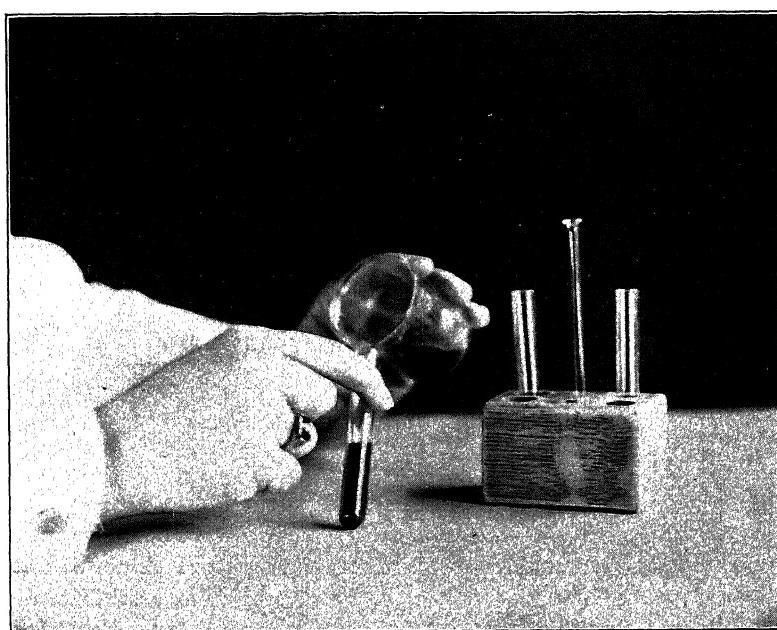
FIG. 125.—FILLING CENTRIFUGE TUBE WITH FECAL SUSPENSION (Benbrook).

results, bright illumination should be obtained by adjusting the mirror and condenser and then modified by closing the diaphragm opening. The microscope should be vertical, not inclined, and the cover-slip area should be searched in a systematic manner in order to see all parts of the drop without going over the same part more than once.

13. When parasite eggs are seen, the high dry power should be used for identification in comparison with illustrations that follow.

De Rivas' Method for Concentrating Ova.—1. Place 5 c.c. of a 5 per cent solution of acetic acid in a test tube with a small amount of feces.

2. Mix well and add 5 c.c. of ether.

3. Shake well and centrifuge at high speed for a minute and examine the sediment for ova.

Pepper's Method for Concentrating Hookworm Ova.—1. Place diluted feces on a slide and allow to remain five minutes.

2. Gently immerse the slide in water.

3. The ova, which have settled to the bottom, cling to the slide and are not washed away.

4. This may be repeated several times.

5. This method is not applicable to ova other than those of the hookworm.

Brine-Flotation Method of Kofoid and Barber.—This method is especially useful when a large number of examinations must be made, especially for the ova of nematodes and cysts of amebae. According to McDonald it is unsatisfactory for trematode eggs, which fail to rise to the surface.

1. A large fecal sample is thoroughly mixed with about twice its volume of saturated solution of table salt in a paraffined pasteboard cup or a small beaker.

2. A lightly compressed circular disk of No. 1 or No. 0 steel wool about one-eighth to one-quarter inch thick is then placed in the cup and pushed to the bottom. This carries down all coarse particles.

3. The fluid is allowed to stand for one hour, during which time the ova rise to the surface.

4. Finally the surface film is looped off with a wire loop about one-half inch in diameter, placed on a slide, and examined without a cover glass. The objective should be focused on the surface of the fluid.

Detection of Tapeworms.—1. Since the head, or scolex, is the ancestor from which the worm is formed in the intestine, it is important, after giving a vermifuge, to make certain that the head has been passed with the worm. Should it remain, a new worm will develop.

2. The technic described by Magath and Brown has proved most satisfactory at the Mayo Clinic. The patient is prepared by abstaining from lunch and supper on the day previous to treatment, although black coffee or tea may be taken freely. At 6 P.M. from 15 to 30 grams magnesium sulphate are taken, and the following morning at 6 A.M. the dose is repeated. Breakfast is not given, and after the bowels have moved, 30 c.c. of the following emulsion is

given: Oleoresina aspidii, 3 grams; acacia (powdered), 4 grams; aquae dis-
tillatae, q.s. ad 30 c.c.   One hour later a second 30 c.c. of the same emulsion
are given.   Two hours later 30 grams of magnesium sulphate are given; two
hours after this a large soapsuds enema is administered.

3. All stools are saved in a container and taken, together with the stool
passed before administration of the drug, directly to the laboratory.

4. To make sure of finding the head, the entire quantity of feces is passed
through a coarse sieve (No. 20) (see Fig. 127).

Detection of Intestinal Amebae.—Three important species are found in
the intestines of man: the *Endamoeba histolytica,* which is definitely patho-

FIG. 126.—LIFTING SURFACE LAYER WITH A BEADED GLASS ROD (Benbrook).

genic, and *Endamoeba coli* and *Endolimax nana,* which may be slightly patho-
genic but are usually considered harmless.

1. If the patient is passing formed stools it is best to collect the specimen
after a saline purge.   During the active stage of the disease (dysentery), the
liquid stool is usually satisfactory or better material may be obtained from
ulcerated areas in the sigmoid collected through the sigmoidoscope.

2. Examine the specimen as soon as possible.   If immediate examination
is not possible, collect in warm container and keep warm until examined.
Specimen should be free of urine.

3. Choose for examination flakes of mucus or bits of tissue.

4. Place the material on a warm clean slide and cover with a cover glass.

5. Examine on the warm stage with low and high objectives, shutting down
the light considerably.

6. If vegetative forms of the ameba are present, they can be distinguished from other cells such as leukocytes by (*a*) their marked motility, (*b*) larger size, (*c*) greenish color, (*d*) hyaline ectoplasm, (*e*) faint nucleus, (*f*) vacuoles, and (*g*) the presence of erythrocytes or other cells in their cytoplasm.

If only cysts are present as in carriers or during the quiescent stage of dysentery, it is better, unless one is thoroughly familiar with their appearance, to use the Donaldson or a staining method described below. Large phagocytic cells or macrophages, which resemble amebae very closely, are often

Fig. 127.—Apparatus for the Recovery of the Heads of Tapeworms.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

present in stools. They are nonmotile and the nuclei are much larger than those of the ameba.

DONALDSON'S METHOD.—1 and 2 as above.

3. Mix a small portion in physiological saline on a slide.

4. To a second portion add a drop of iodine-eosin solution.

IODINE-EOSIN SOLUTION

Eosin (sat. sol. in physiologic saline)............ 2 c.c.
Iodine solution
    Physiologic saline................. 100 c.c. ⎫
    Potassium iodide................   5 gm. ⎬ 1 c.c.
    Iodine to saturation.... .... ⎭
Physiologic saline............................. 2 c.c.

5. Place a cover glass over both drops and examine.

6. All degrees of staining can be found. The amebic cysts gradually take a yellow or brown color and stand out clearly. The nucleus also becomes more clearly defined. Bacteria, yeasts and fecal particles stain pink.

DIAGNOSIS OF THE COMMONER PARASITIC INFESTMENTS OF THE INTESTINES OF MAN

The Intestinal Amebae.—The following table by Todd and Sanford summarizes the characteristics of the three important amebae in the vegetative stage as found in diarrhea and in stools following a saline cathartic (Fig. 128):

Endamoeba Histolytica	Endamoeba Coli	Endolimax Nana
Pathogenic	Nonpathogenic	Nonpathogenic
Size varies with different races of the parasite, between 20 and 40 μ	Size about the same, 20 to 30 μ	Very small, 6 to 12 μ, average 8 μ. Important
Ameboid motion very active in fresh feces. Parasite moves from place to place. Very characteristic	Motion sluggish. Parasite rarely moves from place to place	Motion moderately active in very fresh feces, but soon lost
Ectoplasm hyaline, refractile, sharply differentiated from endoplasm. Characteristic	Ectoplasm slightly refractile and poorly differentiated from endoplasm	Ectoplasm and endoplasm not sharply differentiated
Red blood cells present in endoplasm when stool contains blood. Practically diagnostic. Few or no ingested bacteria	Endoplasm contains many bacteria and fecal particles, but very rarely a red blood cell	Endoplasm contains bacteria, but no red blood cells
Nucleus in living specimen indistinct, often invisible	Nucleus distinct	Nucleus indistinct
Nucleus in specimens stained with iron-hematoxylin shows scanty chromatin arranged in a thin granular ring with a small granule (karyosome) in the center. Diameter 4 to 7 μ	Nucleus richer in chromatin and has a thicker peripheral ring of large granules. Karyosome larger and eccentrically placed. One or more granules of chromatin usually present between karyosome and peripheral ring. Diameter 4 to 7 μ	Nucleus very small, 1 to 3 μ. Chromatin arranged in several small masses connected by bands

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

The following table shows the characteristics of the cysts most frequently found in formed stools. They offer the best means of differentiating the species, especially when studied in fresh condition (Fig. 129).

Endamoeba Histolytica	Endamoeba Coli	Endolimax Nana
Cysts, spheric or slightly ovoid Cyst wall single and delicate in young cysts, thicker and sometimes double outlined in older ones	Similar, but double outline of wall more frequent and much more distinct Entire cyst more refractile	Cyst definitely ovoid, thin-walled
Size differs with different races of the parasite, 7 to 15 μ	Usually large, 15 to 22 μ	Small, 7 to 9 μ
Cytoplasm of one- and two-nucleated cysts granular, often with a poorly defined clear area which stains brown with iodine (glycogen) Presence of chromidia (brightly refractile spindle-shaped or irregular masses of chromatin) characteristic Glycogen and chromidia disappear as cyst matures	Similar, but glycogen more abundant and sharply defined when stained with iodine. Chromidia rare	Glycogen sometimes present as a single mass, staining deeply with iodine Chromidia absent
Fully developed cysts contain four small nuclei seen by focusing at different levels	Fully developed cysts contain eight to sixteen nuclei, eight being the normal number The four-nucleate stage of developing cyst rarely seen	Fully developed cysts contain four, very rarely eight, very small nuclei
With iron - hematoxylin the nuclei, although much smaller, show the same structure as in vegetative stage	Structure of nuclei same as in vegetative stage	Structure of nuclei same as in vegetative, but difficult to see owing to their small size

Balantidium Coli.—This parasite, formerly called *Paramecium coli,* is an occasional inhabitant of the colon of man, where it penetrates into the mucous membrane and produces a diarrheal condition resembling amebic dysentery. Infection is most frequent among farmers, and in some cases has been associated with the symptoms of pernicious anemia. It is an actively moving oval organism, about 60 to 100 micra long, and 50 to 70 micra wide, is covered with cilia, which are arranged in longitudinal rows giving a striated appearance, and contains a bean-shaped macronucleus, a globular micronucleus, two contractile vacuoles, and variously sized granules (Fig. 130). At the anterior end is a funnel-shaped mouth. The parasite is so large that it can hardly be overlooked if present upon the slide and still active. Its ordinary habitat is the large intestine of the domestic pig, where it apparently causes no disturbance. It probably reaches man in the encysted condition.

Taenia Saginata.—This is a common tapeworm or cestode and is called the beef tapeworm. Segments are usually found (see Fig. 127). The ova are spheric or ovoid, yellowish-brown in color and with a thick, radially striated cortex usually surrounded by a broad transparent and slightly granular zone called the vitelline membrane (Fig. 131).
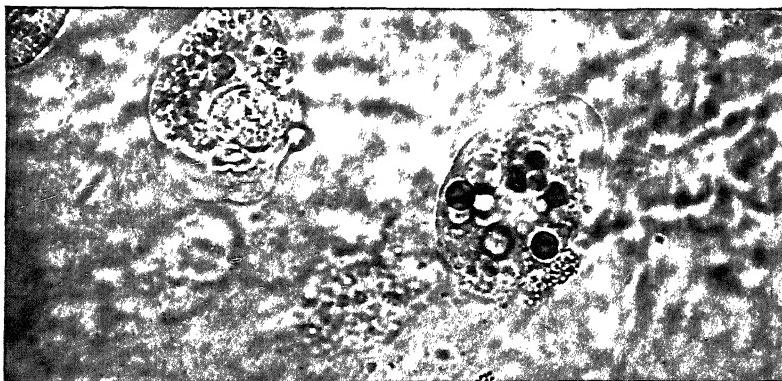
FIG 128—ENDAMOEBA HISTOLYTICA

Motile forms showing ingested red cells and clear ectoplasm   (After Army Medical School Collection, Washington, D C)

FIG 129—ENDAMOEBA COLI CYST SHOWING A LARGE VACUOLE.
(After Army Medical School Collection, Washington, D C)

Dibothriocephalus Latus.—This is the "fish" tapeworm. The ova are brown (45 to 65 micra) and filled with spherules   The shell is thin with a small hinged lid at one end (Fig 132).

Hymenolepis Nana.—The ova are colorless, semitransparent, nearly round, and contain an embryo surrounded by two distinct membranous walls between which is a broad gelatinous substance (Fig 133).
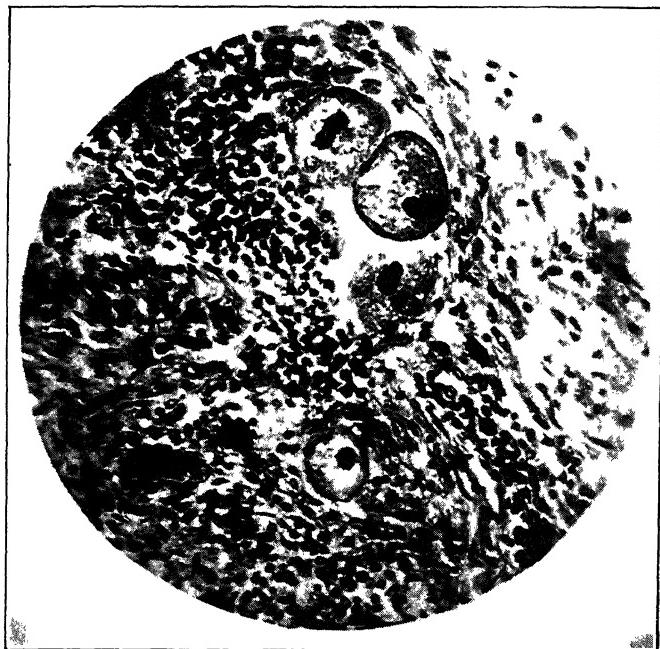
FIG 130—BALANTIDIUM COLI

Several balantidia deep in tissue of colon associated with infiltration of lymphocyte (After Strong)
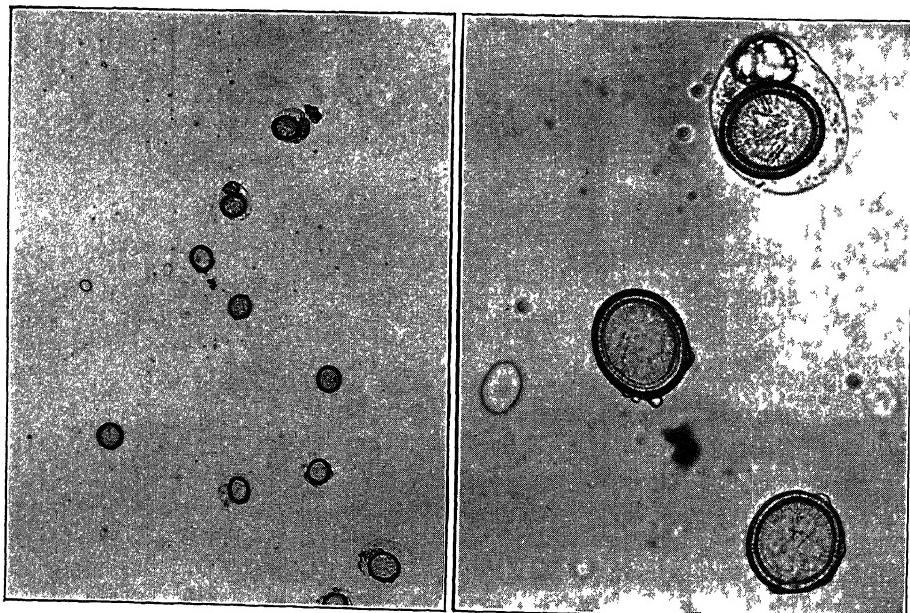
FIG 131—OVA OF TAENIA SAGINATA (Benbrook) × 100 and × 400.

Taenia Echinococcus.—Diagnosis is made by finding the scolex and hooklets in cyst fluids, by the complement-fixation reaction and by precipitin and skin tests.

Ascaris Lumbricoides.—This is a common "round" worm   The ova are elliptic (45 to 50 by 60 micra), yellow-brown in color, and have an unsegmented protoplasm   There is usually a crescentic clear space at each pole, between the contents and the shell (Fig 134)   The shell is moderately thick and smooth, and is covered with an irregular albuminous coating which gives it a roughly sculptured surface Sometimes this coating is lost and the surface of the shell is then smooth   When only females are present in the intestine, and occasionally at other times, one finds unfertilized eggs   These are generally
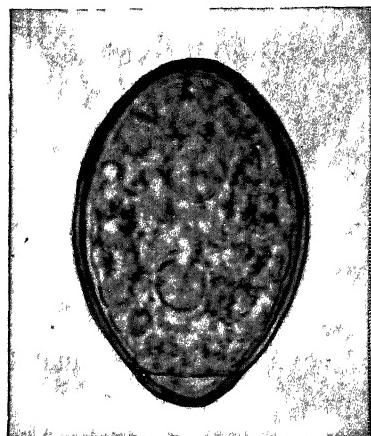
FIG 132—OVA OF DIBOTHRIO-
CEPHALUS LATUS   (Wood)
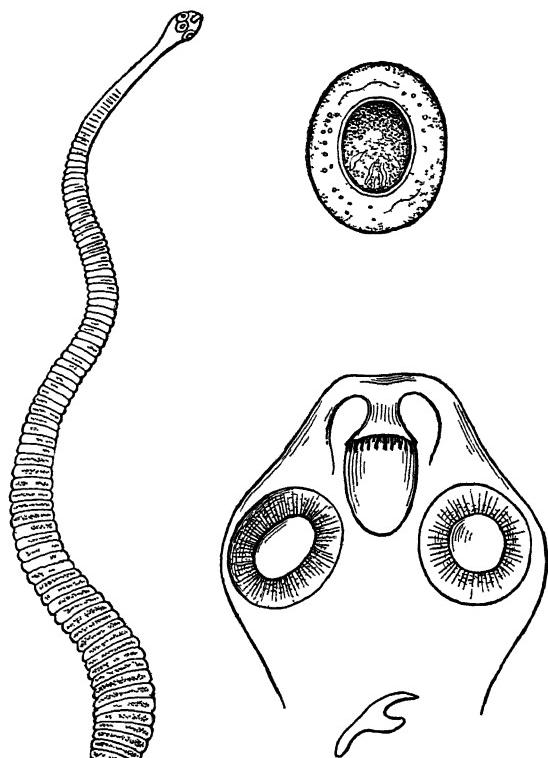
FIG 133—HYMENOLEPSIS NANA
Parasite to the left, egg, scolex and hooklet to
right   (After Braun)

more elongated, have a thinner shell, and are filled with coarse granular contents which obliterate the crescentic clear spaces at the ends   Many are roughly globular and some are so extremely irregular in outline as to bear little resemblance to an egg   Such unfertilized ova have doubtless many times passed unrecognized even by clinical microscopists of some experience.

Oxyuris Vermicularis.—This is the "pin" or "seat" worm.   The worms are very small (female from 9 to 12 millimeters long; male about 3 to 5 millimeters) but may be found by placing portions of stool in clean water in a Petri dish held against a black background   The ova are colorless and asym-

metrically oval with one flattened side, are about 50 micra long by 16 to 25 micra wide, have a moderately thin, double-contoured shell and when deposited contain a partially developed embryo (Fig. 135). The diagnosis is best made by giving a purgative or a copious enema and searching the stool for the adult worms.
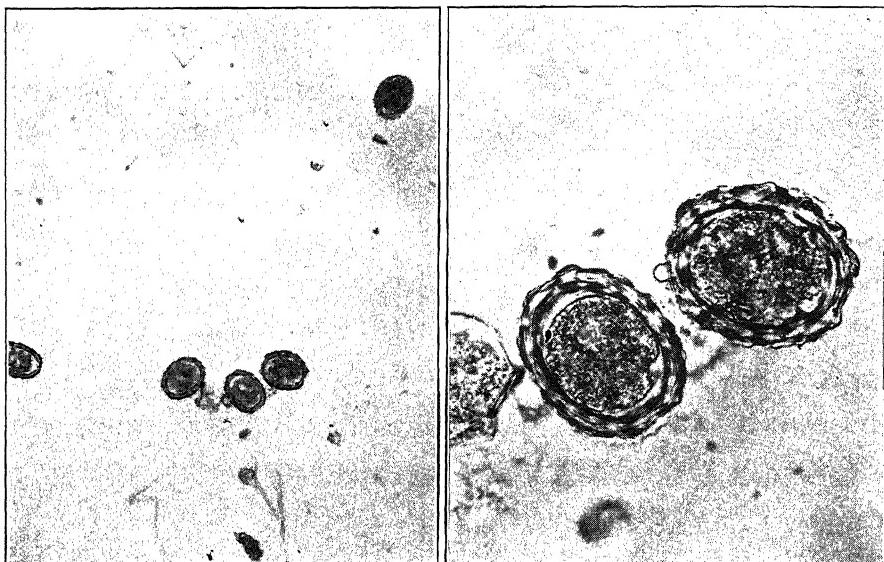
FIG. 134.—OVA OF ASCARIS LUMBRICOIDES (Benbrook). X 100 and X 400.

Ankylostoma Duodenale and Necator Americanus.—The former is the common hookworm of Europe and the latter of America. They are also called *Uncinaria.* The ova are oval and have a thin, smooth, transparent shell. As they appear in the feces the protoplasm is divided into 2, 4, 8 or more rounded segments. They measure 32 to 38 by 52 to 62 micra (Fig. 136).
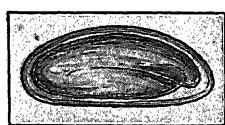
FIG. 135. — OVUM OF OXYURIS VERMICULARIS. X 460. (Wood.)

Strongyloides Stercoralis.—The larvae are actively motile, with a striking "wriggling" motion, and, when the stool is solid, are best found by making a small depression in the fecal mass, filling it with water, and keeping in a warm place (preferably an incubator) for twelve to twenty-four hours. The larvae will collect in the water, and can be easily found by transferring a drop to a slide and examining with a 16 millimeter objective. The inexperienced worker should *make sure that worms move,* or he may be misled by the vegetable hairs (notably those from the skin of a peach) closely resembling small worms. The ova are shown in Figure 137.

Trichuris Trichiura.—This is the common "whip" worm. The ova are brown, ovoid, 50 by 54 micra long and about 23 micra wide, and have a button-like projection at each end (Fig. 138).

FIG. 136.—OVA OF NECATOR AMERICANUS OR AMERICAN HOOKWORM (Benbrook).
× 100 and × 400.
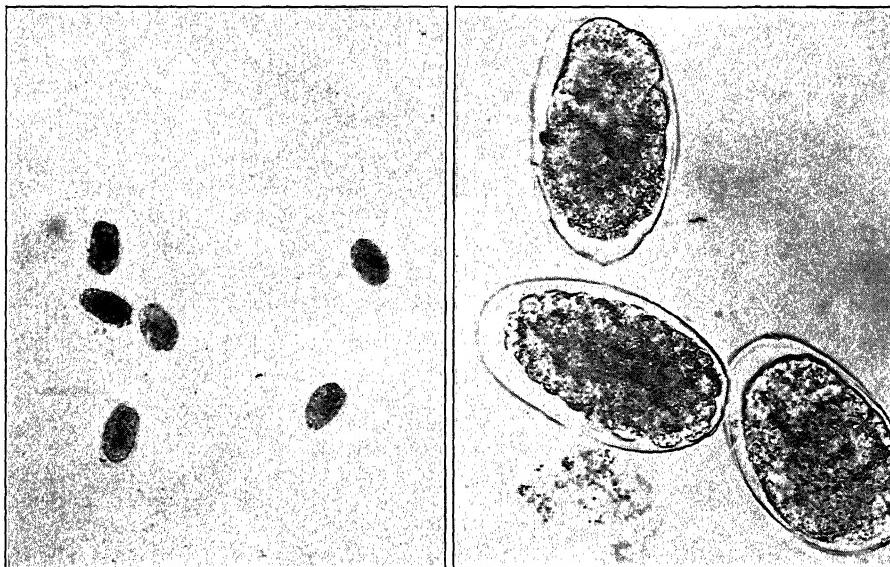
FIG. 137.—OVA OF STRONGYLOIDES STERCORALIS (Benbrook). × 100 and × 400.

Trichinella Spiralis.—This parasite produces trichiniasis and human beings are usually infested by eating pork containing the encysted larvae. The blood generally shows an eosinophilia. The larvae are liberated in the intestines and

develop into worms. The males die but the females burrow into the intestinal mucosa and produce large numbers of embryos which are carried by the blood
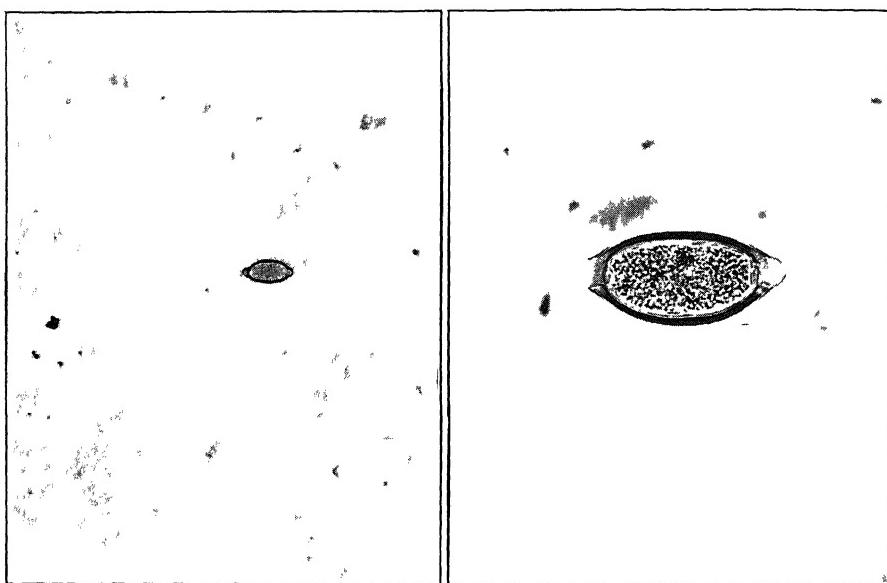
FIG. 138.— OVA OF TRICHURIS TRICHIURA (Benbrook). × 100 and × 400.

to striated muscle. Diagnosis is made by teasing out upon a slide a bit of muscle, obtained in man preferably from the pectoralis major, the outer head

of the gastrocnemius, the insertion of the deltoid, or the lower portion of the biceps In the case of rats, the diaphragm, which is the most likely site, is pressed out between two glass slides. The coiled larvae can easily be seen with a 16 millimeter objective (Fig. 139). The larvae can usually be found in the spinal fluid and the blood before they have reached their final resting place in the muscles. During the diarrheal stage, adult worms may be present in the feces, and can sometimes be found by diluting with water, decanting several times, and examining the sediment in a very thin layer in clean water with a hand lens.

FIG. 139 —TRICHINA SPI-
RALIS ENCYSTED IN
MUSCLE. (Wood)

Fasciola Hepatica.—This "liver fluke" is common in sheep and occasionally infests man. Ova appear in the feces and are yellowish-brown, oval, operculated, and measure about 130 to 140 by 75 to 90 micra (Fig. 140).

Trichomonas Hominis.—This flagellate is pear-shaped and presents four flagella anteriorly and an undulating membrane directed from the anterior end
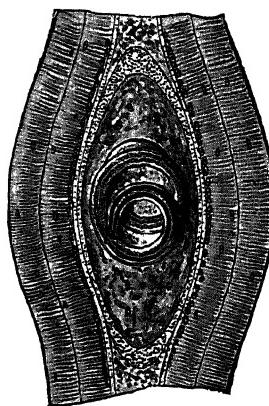
posteriorly over the surface of the body. It measures about 9 by 15 micra. It exhibits a peculiar undulating movement and is quite active for hours. It is not known to become encysted. It occurs in the large intestine and similar trichomonas occur in the vagina and the mouth. It is easily recognized in feces with the low-power objective. A drop of Gram's iodine added to the preparation kills them and the structures are more clearly seen (Fig. 141).

Chilomastix Mesnili.—This parasite is often confused with *Trichomonas hominis* on account of somewhat similar form and action. It has three anterior flagella and a fourth short flagellum directed posteriorly through a slitlike
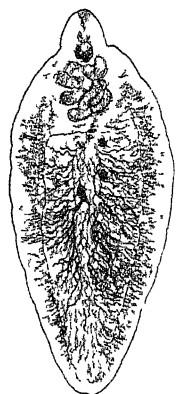
FIG 140—FASCIOLA
HEPATICA   × 5.
(Wood)
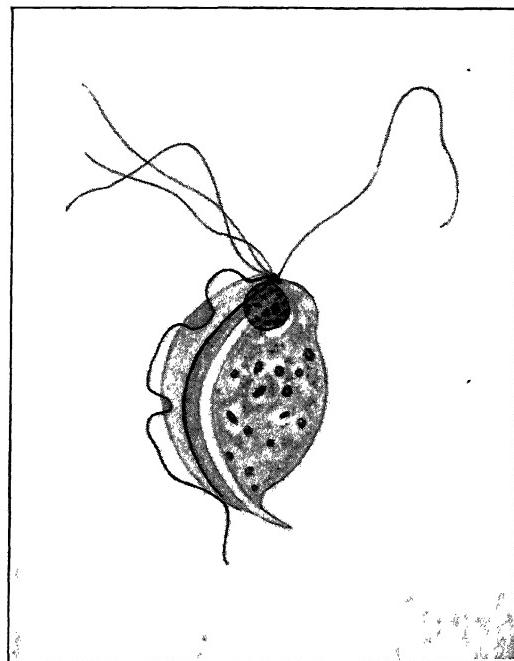
FIG 141—TRICHOMONAS HOMINIS
(After Dobell and O'Connor)

cytostome in the anterior end. It inhabits the large intestines. Methods for detecting are the same as for *Trichomonas*

Giardia Lamblia.—This flagellate is pear or tennis racket in shape with a depression at the blunt anterior end. Three pairs of flagella occur around the depression and one pair projects from the small tail-like extremity. Size, 10 by 20 micra. It is very active and exhibits a tumbling movement. Its usual habitat is the duodenum and its presence is often detected by biliary drainage.

The vegetative forms are not usually found in the feces unless a purgative has been given. They become encysted and the cysts are passed in the stool. The diagnosis is often made by finding the cysts in the stools. The cysts are oval, measuring 8 by 14 micra (Fig. 142).

Schistosoma Haematobium.—The ova of this trematode, frequently called *Bilharzia haematobia,* occur in abundance in the mucosa of the bladder and rectum and may be found in the urine and feces. They are elongated, oval,

FIG. 143.—OVUM OF SCHISTOSOMA JA-PONICUM.   × 460, (Morris.)

A

B
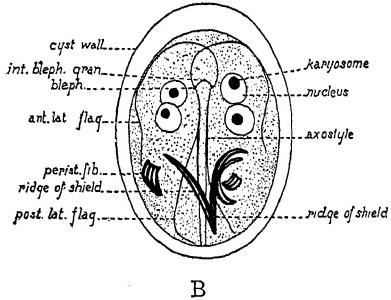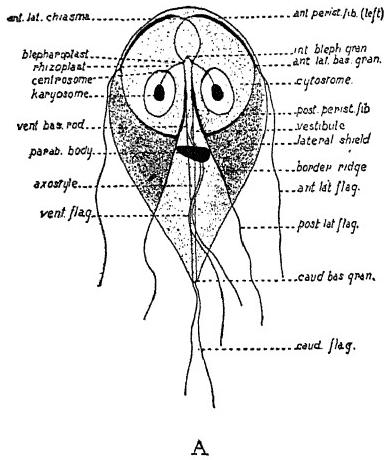
FIG. 142.—GIARDIA LAMBLIA.
A. Flagellated stage. B. Cyst.
(After Simon.)
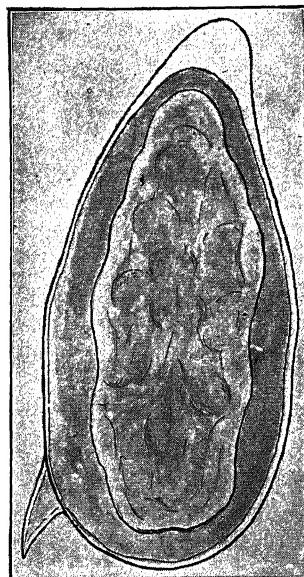
FIG. 144.—OVUM OF SCHIS-TOSOMA MANSONI. × 460.
(Morris.)

about 120 to 190 micra long, and 50 to 73 micra broad, yellowish in color, and slightly transparent. They possess no lid such as characterizes the eggs of most of the trematodes, but are provided with a thornlike spine which is placed at one end. Within is a ciliated embryo.

Schistosoma Japonicum resembles *Schistosoma haematobium* morphologically, but both the male and female are smaller. The ova (Fig. 143), which appear in the feces, are ovoid, thin-shelled, and without lid or spine. They average 83 by 62 micra in size, and contain a ciliated embryo. The worm inhabits the portal, and probably also other veins. A fresh-water snail (some species of Planorbis) serves as intermediate host. The rice fields are often the place of infection.

Schistosoma Mansoni.—The adult worms closely resemble the male and female of *Schistosoma haematobium*. They inhabit the rectal and portal veins, and ova appear in the feces, where they are very easily recognized from their size and the characteristic spine (Fig. 144). They are light yellow in color, measure 112 to 162 by 60 to 70 micra, and are provided with a clean-cut, sharply pointed spine, which is situated at the juncture of the last and third quarters of the egg, and is directed backward. Within the egg is a ciliated embryo (miracidium) which can be seen without difficulty. The life history is very similar to that of *Schistosoma haematobium*.

CHAPTER XIII

METHODS FOR THE EXAMINATION OF EXUDATES AND TRANSUDATES

Principles.—1. *Transudates* are the result of noninflammatory processes and are usually due to disturbances of circulation with passive congestion and edema. The most familiar examples are pleural, pericardial and peritoneal effusions, the last named being called "ascites." The cerebrospinal fluid is also in part a transudate but is separately considered. Transudates are light yellow or serous in appearance and sometimes turn yellowish-green upon standing. At times they are milky or reddish and always of about the alkalinity of the blood.

2. *Exudates* are the result of inflammatory processes usually due to bacterial infection. As a result they vary greatly in appearance and are usually richer in cells and coagulable materials. They are likewise usually alkaline in reaction. Various types have been classified, as follows:

(a) Serous: straw color; contains but few cells
(b) Fibrinous: yellow; rich in fibrinogen; coagulates
(c) Purulent: pus of various colors
(d) Hemorrhagic: contains blood
(e) Chylous: contains fat globules; milky
(f) Chyloid: also milky but contains a complex of pseudoglobulin and lecithin with some fat
(g) Putrid: usually associated with gangrene
(h) Mixed: combinations of the above are most common

3. Aspirated fluids should be examined as soon as possible after removal. Small amounts are not sufficient for complete examinations including specific gravity, cytologic studies and guinea-pig inoculation; at least 150 to 250 c.c. should be removed whenever possible.

ROUTINE EXAMINATION

This usually embraces the following for differential diagnosis, as no one method of examination can be used alone; a combination of procedures is advisable.

1. Appearance.
2. Specific gravity, estimated according to the method employed in urine analysis (see Chapter VI).

3. Presence or absence of partial or complete coagulation and rapidity of coagulation. Exudates coagulate more rapidly and completely than transudates. The latter may not coagulate at all or show flocculi, whereas some exudates (notably those obtained from the lungs by bronchoscopic drainage) may coagulate solid or show partial coagulation.

4. Chemical determinations, especially for quantity of protein.

5. Examination of cells (cytodiagnosis).

6. Bacteriological examination (see Chapter XIX) by smear, culture and animal inoculation (in suspected tuberculosis).

7. Complement-fixation tests in some instances for tuberculosis, syphilis and echinococcus disease (see Chapter XXVII).

8. The following table summarizes the usual differential properties of transudates and exudates.

Tests	Transudates	Exudates
Specific gravity	1.006 to 1.015 (average about 1.013). Tumor transudates, 1.018 to 1.025	Over 1.018, with average about 1.022
Coagulation....	Usually absent or slight	Usually positive
Protein..	Rivalta test usually negative. May be positive after concentration of fluid by absorption. Under 3 per cent in quantity	Rivalta test usually positive. Over 3 per cent in quantity
Cytology..........	Endothelial cells and erythrocytes. Small lymphocytes sometimes predominate. Tumor cells may be found. Eosinophils may be increased after repeated tappings	Polymorphonuclears in acute infections. Small lymphocytes in chronic infections. Eosinophils in pneumococcus infections, after repeated tappings and following artificial pneumothorax. Erythrocytes usually present
Bacteriology............	Usually sterile. Staphylococcus albus from the skin may occur in cultures	Smears and cultures usually positive for pneumococci, streptococci, etc. Tubercle bacilli in smears and by guinea-pig inoculation
Complement-fixation	Positive reactions in syphilis	Positive reactions in tuberculosis and echinococcus disease

CHEMICAL EXAMINATION

1. The protein content is usually proportional to the specific gravity, that is, higher in exudates than in transudates.

2. Exudates usually show about three times as much albumin, globulin and fibrin as transudates. Albumin-globulin ratios are not sufficiently constant for differentiation.

3. Nonprotein nitrogen, urea nitrogen, creatinine, sugar, uric acid, chlorides, inorganic phosphorus and total calcium usually parallel these substances in the blood. In tumor cases the uric acid may be higher than in the blood, probably because of increased destruction of nucleoprotein. Cholesterol varies considerably.

4. Methods of quantitative estimation of these substances are the same as employed in Chapter XXXI for blood analysis. For the proteins alone the following tests may be employed for differentiating between exudates and transudates.

Qualitative Acetic Acid Test (Rivalta).—1. Place about 200 c.c. of distilled water in a conical glass.

2. Add 2 drops of glacial acetic acid.

3. Mix thoroughly.

4. Allow 1 or 2 drops of the puncture fluid to fall into this weak acid solution.

5 A distinct cloud will be observed in the wake of the falling drop if the fluid is an exudate. As a rule if the fluid is a transudate no turbidity will be noticed. The reaction is probably due to the large amount of globulin present in exudates. Positive reactions may occur with transudates concentrated by absorption or those developing after tapping and the production of artificial pneumothorax.

Quantitative Protein Determination.—1. The protein content may be determined by the Esbach method for the quantitative estimation of protein in urine.

2. Owing to relatively large amounts of protein likely to be present it is advisable to test the fluid diluted 1 : 2, 1 : 5 and 1 : 10 with saline solution The measure of precipitate is multiplied by 2 5 and 10 respectively

3. The results show grams per liter. Divide by 10 to obtain the per cent.

DIFFERENTIAL CELL COUNT

(*Cytodiagnosis*)

1. Centrifuge *fresh* (important) specimen of the fluid. To prevent coagulation, the fluid may be collected in a little sodium citrate solution, although cytodiagnosis is better made without the use of anticoagulants

2. Pour off supernatant fluid and make thin smears of sediment on slides It is essential to use packed sediment in order to secure sufficient cells in smears.

3. Dry in the air.

4. Stain with Wright's or Giemsa's stain according to the method of staining blood smears.

5. Count and tabulate at least 100 of the cells. Four types may be present: lymphocytes, polymorphonuclears, eosinophils and endothelial cells. Erythrocytes in varying numbers are usually present in all fluids.

6. Sections of imbedded sediment are especially valuable when malignancy is suspected. Centrifuge the fluid in a conical centrifuge tube. Pour off the
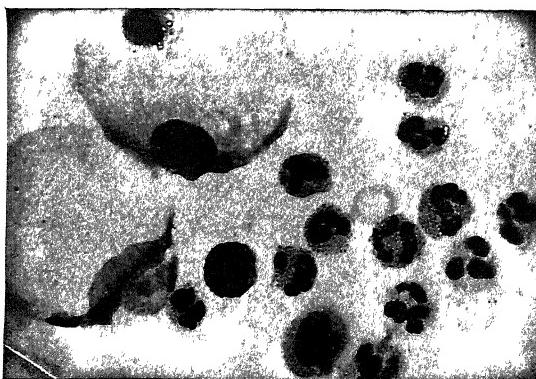
FIG 145—CYTODIAGNOSIS

Excess of polymorphonuclears   (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W B Saunders Co)

supernatant fluid and add more fluid. Centrifuge again and continue in this manner until 3 to 5 c c. of sediment have been obtained. After pouring off the last supernatant fluid, imbed, section, and stain the packed sediment according to methods given in Chapter XXXVI.

Interpretation.—1. The types of cells found depend on (*a*) the primary etiological factor; (*b*) the stage of severity of the etiological process; (*c*) the duration of the effusion, and (*d*) the secondary factors and especially the number of tappings.

2. Polymorphonuclear neutrophil leukocytes predominate in acute infectious processes, especially those due to the pyogenic organisms (Fig. 145), and may be found in early acute cases of serous tuberculous exudates.

3 *Small lymphocytes* predominate in chronic processes, especially those due to tuberculosis and syph-

FIG 146—CYTODIAGNOSIS

Excess of small lymphocytes. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W B Saunders Co.)

ilis (Fig. 146). They may also predominate in some chronic nontuberculous pleurisies, chronic transudates, or even tumor transudates.

4 *Eosinophils* may be present but have no clinical significance except to

suggest an allergic origin of the fluid or disease due to an animal parasite. Serous effusions caused by pneumococci may contain as high as 10 per cent eosinophils, and, according to Foord, a frequent reason for their presence is repeated aspirations when they may be as high as 75 per cent.

5. *Endothelial cells* (Fig. 147) in large numbers along with lymphocytes and erythrocytes are usually present in transudates and are largely derived from the endothelium lining the large serous cavities (pleural, peritoneal, pericardial). They commonly occur in sheets as well as singly.

6. The presence of masses of large cells, irregular in size and shape, often vacuolated, showing prominent nucleoli and sometimes mitotic figures, is highly
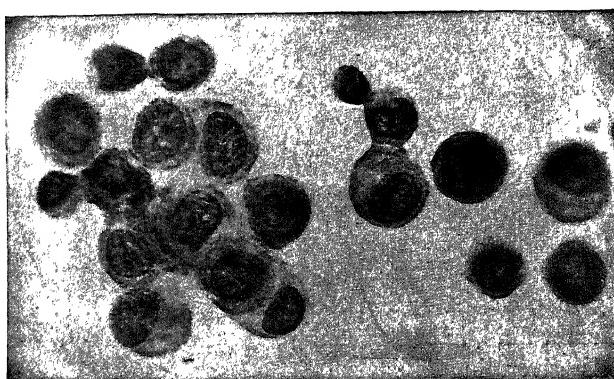
FIG. 147.—CYTODIAGNOSIS.

Endothelial cells. (From Todd and Sanford. *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)

suggestive of malignancy, but definite diagnosis is better made by sections of the imbedded sediment, in which fragments of tumor tissue, especially gland acini in adenocarcinomata, can be sometimes found. Confusion may result in smear examinations when degenerated forms of large mononucleated cells, either serosal desquamations or cells of other types ordinarily designated as macrophages, are seen.

BACTERIOLOGICAL EXAMINATION

1. These methods are described in more detail in Chapter XIX.

2. Cultures should be made on blood agar or in hormone broth. Relatively large amounts of fluid, like 1 c.c., should be employed.

3. Smears of sediment should be stained by the Gram method and for tubercle bacilli.

4. Prolonged microscopic examination is usually required for the detection of tubercle bacilli.

5. In conducting the inoculation test for tubercle bacilli, at least several ounces of fluid should be centrifuged and the sediment injected into guinea-pigs. The injection of 5 c.c. amounts of fluid may yield falsely negative results.

BACTERIOLOGICAL CONTROL OF TREATMENT OF INFECTED WOUNDS

1. There should be no disinfectant treatment of the wound for at least two hours before smears and cultures are made.

2. Every two or three days smears should be made on slides with a sterile platinum wire or sterile swabs, care being taken to obtain material from the worst parts of the wound.

3. Dry in the air. Fix with heat. Stain with methylene blue or by Gram's method.

4. Wash with water, dry and examine (with oil-immersion lens and No. 10 ocular).

5. Estimate the average number of bacteria per field. The results may be charted.

6. As long as there are over 50 bacteria per field, closure of the wound is contra-indicated.

7. If 50 or less, make the examination each day and likewise cultures on blood agar for hemolytic streptococci.

8. If smears show gram-positive bacilli resembling the anaerobes, make anaerobic cultures.

9. The presence of hemolytic streptococci in any number contra-indicates closure.

10. The presence of a few saprophytes (2 or 3 per field) does not contra-indicate secondary suturing of the wound.

11. Cultures made from wounds recently treated with a chlorine compound may be freed from the antiseptic chlorine by obtaining the material on a sterile swab, immersing this in a tube of sterile N/10 sodium thiosulphate solution, and then preparing cultures from this neutralized mixture.

CHAPTER XIV

METHODS FOR THE COLLECTION AND EXAMINATION OF CEREBROSPINAL FLUID

COLLECTION

1. Spinal puncture for the collection of cerebrospinal fluid may be conducted in an office or laboratory, but is better done in a hospital or the home
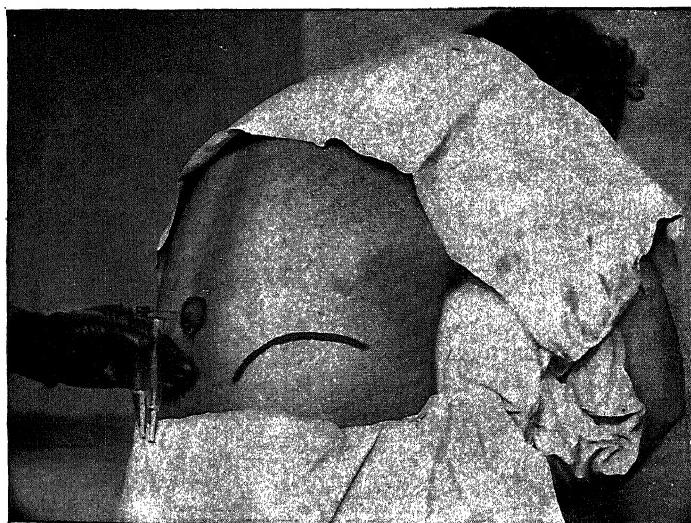
FIG. 148A.—SPINAL PUNCTURE IN THE SITTING POSTURE.
(From Keen's *Surgery*.)

of the patient, since it is advisable for the patient to rest in bed for at least eighteen hours immediately after the puncture as a safeguard against spinal puncture headache.

2. The *needle* should not be too large, in order to reduce pain to a minimum and to inflict the minimum of damage to the meninges. Gauge No. 19 is about right, unless acute suppurative meningitis is suspected, in which case No. 15 may be used if a purulent and thick fluid is present. The needle should be sterilized just before use and should be perfectly straight and sharp with a short bevel. Crooked, rusty, dull and unnecessarily large needles are the usual causes of failure and the infliction of unnecessary pain.

3. The *sitting posture* may be used in the puncture of ambulatory adults, as shown in Figure 148A, but the reclining posture with the patient lying on
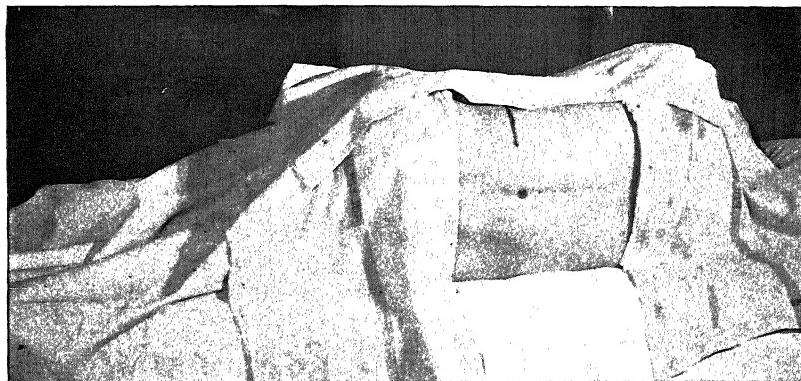
FIG. 148B.—SPINAL PUNCTURE IN THE PRONE POSITION WITH THE BACK WELL ARCHED AND PERPENDICULAR TO THE TABLE.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

his right side (Fig. 148B) is recommended, especially if the spinal fluid pressure is to be taken. The latter is required in the case of children and sick adults.

FIG. 149.—PRODUCING LOCAL ANESTHESIA.
(From Keen's *Surgery*.)

4. The skin should be carefully disinfected with tincture of iodine followed by alcohol. The hands of the operator should be likewise carefully cleansed

and the use of sterile rubber gloves is recommended.   The operative field should be protected with sterile sheets and towels.

5. With adults the puncture can usually be made without an anesthetic. The skin may be infiltrated with sterile 1 per cent novocaine or butyn solution (Fig. 149).   Struggling children and adults may require a few drops of chloroform as it is dangerous to conduct the puncture under such conditions since the needle may be broken.
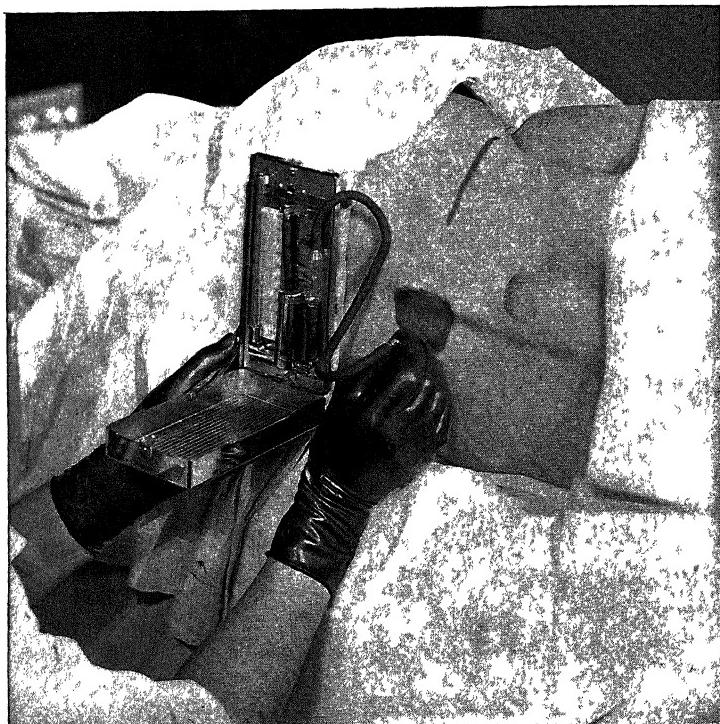
FIG 150—MEASURING SPINAL FLUID PRESSURE WITH A MERCURY MANOMETER
(From Kolmer, *Chemotherapy*, W B Saunders Co)

6. Puncture is best conducted between the fourth and fifth or between the third and fourth lumbar vertebrae.

7. The "soft spot" between the spinous processes is located and the needle *gently and slowly* passed in the middle line.   The distance varies according to the age and weight.   A peculiar "give-way" sensation to the needle denotes entrance into the subarachnoid space, or during its passage the stylet may be removed from time to time to determine whether or not it has entered sufficiently as shown by a flow of fluid.

8 If pure blood is obtained, the needle should be withdrawn and the needle cleansed or the puncture repeated with a fresh needle.

9 If there is no flow of fluid the needle may be gently turned or slightly withdrawn or entered a little further. "Dry taps" are usually due to the fact that the needle has not entered the subarachnoid space.

10. The pressure (if to be taken) should be taken before the escape of fluid (Fig. 150)

11. *Fluid should be collected in two sterile tubes,* one of which (No. 2) may contain a trace of powdered potassium oxalate to prevent coagulation. From 3 to 5 c c. may be collected in No 1 to be used for culture and the Wassermann test even if it is slightly blood-tinged A similar amount may be collected in No. 2 to be used for the total and differential cell counts, protein and sugar determinations and the colloidal tests (gold, mastic or benzoin). This fluid should be free of blood

12 The needle is now quickly withdrawn, the iodine removed from the skin and the puncture sealed with flexible colloidin or with an aseptic dressing. The patient should rest on the back for at least half an hour and preferably stay in bed for at least eighteen hours to reduce the chances of developing spinal puncture headache, which is believed to be due to the continued leakage of spinal fluid into the epidural space (hence the advisability of using as small a needle as possible and of reaching the fluid at the first puncture).

GENERAL PHYSICAL EXAMINATION

1. **Color.**—Normal spinal fluid is perfectly colorless like distilled water. Color may be recorded as follows:

(*a*) Colorless
(*b*) Yellow or yellowish (xanthochromia) due to altered
   hemoglobin, jaundice or such drugs as acriflavin)
(*c*) Red or reddish (erythrochromia) due to blood or hemoglobin
(*d*) Greenish, grayish, etc., in meningitis

2. **Transparency.**—Normal spinal fluid is perfectly clear and transparent like distilled water. A single drop of blood in 5 c c may, however, render it opalescent. A classification may be made as follows:

(*a*) Perfectly clear
(*b*) Faintly opalescent (detected by viewing the tube against a black
   background)
(*c*) Distinctly opalescent
(*d*) Faintly turbid
(*e*) Markedly turbid
(*f*) Purulent

3 **Coagula and Sediments.**—Normal spinal fluid does not coagulate. In acute and chronic meningitis or passive congestion, fibrinogen may be present,

which changes into fibrin with coagulation after standing. The following terminology is recommended:

(a) No coagula

(b) Numerous small coagula (as in paresis)

(c) "Cobweb" or "pine-tree" coagulum (typical of tuberculous meningitis)

(d) Heavy sunken coagulum and sediment (as in acute suppurative meningitis)

4. **Specific Gravity.**—This is not usually included in an ordinary examination. The normal varies from 1.006 to 1.008. It may be taken by the methods described for the specific gravity of urine.

5. **Reaction.**—Normally the spinal fluid is slightly alkaline with a P^H of about 8.11 as compared to 7.6 to 7.8 of the blood. The reaction is not generally taken as part of a routine examination.

6. **Freezing Point.**—Normally from −5.51 to −0.58° C. Not usually taken in routine examinations.

TOTAL CELL COUNT

Principles.—1. Whenever possible the total cell count should be made immediately after the collection of fluid while the cells are in suspension and before coagula have formed. If there is no excess of fibrin, so that coagulation does not occur, counts made some hours later or next day are fairly accurate, providing the fluid is well shaken to secure an even resuspension of cells. By collecting fluid in a tube carrying a minute amount of potassium oxalate as described above, coagulation is prevented and counts made hours later compare quite closely with those made immediately after collection.
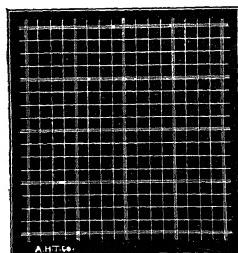
FIG. 151.—FUCHS-ROSENTHAL RULING.

2. Great care in technic and accuracy are recommended because the total cell count possesses a considerable degree of diagnostic value in syphilis, acute anterior poliomyelitis and lethargic encephalitis where slight increases may occur, detectable only by accurate counts. When there is a considerable increase of total cells, as in the different types of acute suppurative and tuberculous meningitis, slight errors in counting have no particular influence upon diagnosis.

3. Spinal fluids containing visible amounts of blood are unfit for total cell counts because of the presence of leukocytes resulting in counts that are too high. Traces of blood too small for naked eye detection also increase the count very slightly but probably not to the point where the error seriously interferes with diagnosis.

Procedure.—1. The Levy counting chamber with the Fuchs-Rosenthal ruling (Fig. 151) is recommended. With the cover glass on it has a depth of 0.2 millimeter with a capacity of a trifle more than 3 c.mm.

2. Draw diluting fluid to the mark 1 in the Thoma or Trenner leukocyte-counting pipets; draw spinal fluid to the mark 11.

<div align="center">DILUTING FLUID</div>

$$\begin{array}{lr} \text{Methyl violet}\ldots\ldots\ . \quad \ldots\ldots\ldots\ . \quad \ldots\ldots\ . & \text{0.2 gm.} \\ \text{Glacial acetic acid}\ldots\ \ldots\ldots\ \ldots\ \ldots\ldots\ldots\ \ldots\ . & \text{10.0 c.c.} \\ \text{Water (distilled)}\ldots\ldots\ldots\ldots\ldots\ldots\ . \quad \ldots\ \ldots & \text{90.0 c.c.} \end{array}$$

Filter. Should be crystal clear and free of artefacts.

3. Shake well as in leukocyte counting and discard 2 or 3 drops.

4. Fill the chamber as in leukocyte counting and wait five minutes for the cells to settle.

5. Count all of the cells (erythrocytes are hemolyzed) in the entire ruled-off space of 16 squares.

6. Calculate as follows:

(a) If the Thoma pipet is used:

$$\frac{\text{number of cells in 16 squares} \times 100}{288} = \text{number of cells per c.mm. of undiluted fluid (factor 0.35)}$$

(b) If the Trenner pipet is used:

$$\frac{\text{number of cells in 16 squares} \times 100}{304} = \text{number of cells per c.mm. of undiluted fluid (factor 0.33)}$$

(c) Or, with either pipet, multiply the cells counted in 16 squares by 11 and divide by 32 to give number of cells per c.mm. of undiluted fluid.

7. Total cell counts can also be made with the ordinary blood-counting chamber (improved Neubauer ruling), although the results are not as accurate. The cells are counted in the entire ruling (*i.e.,* in 9 sq. mm.) and the calculations made as follows:

(a) Using the Thoma pipet:

$$\frac{\text{number of cells} \times 100}{81} = \text{number of cells per c.mm. of undiluted fluid (factor 1.23)}$$

(b) Using the Trenner pipet:

$$\frac{\text{number of cells} \times 100}{171} = \text{number of cells per c.mm. of undiluted fluid (factor 1.17)}$$

8. If a double chamber is used, a simple and approximately accurate method

with either the Thoma or Trenner pipets consists in counting the cells in 10 1-millimeter squares (5 in each ruling) and disregarding the dilution. The result will be the number of cells per c.mm. of undiluted fluid.

9. Total cell counts are interpreted as follows:

Normal: o to 8 per c.mm. undiluted fluid
Border line: 10 to 12 per c.mm. undiluted fluid
Slight increase: 15 to 30 per c.mm. undiluted fluid
Moderate increase: 30 to 100 per c.mm. undiluted fluid
Great increase: hundreds to thousands per c.mm. undiluted fluid

DIFFERENTIAL CELL COUNTING AND CYTODIAGNOSIS

1. Centrifuge fresh specimen of fluid.

2. Pour off supernatant fluid and make thin smears of sediment on slides. Or tease out coagula on slides.

3. Dry in air.

4. Stain with Wright's or with methylene blue stain.

5. Count and tabulate cells (lymphocytes, polymorphonuclears and endothelial cells) as described in Chapter XIII and determine the number of each variety per 100 cells.

6. Normally, only lymphocytes and occasional endothelial cells are found.

7. In acute suppurative meningitis due to the pneumococcus, meningococcus, streptococcus, etc., polymorphonuclear cells predominate in acute stage.

8. In tuberculous meningitis, small lymphocytes predominate (usually).

9. In acute anterior poliomyelitis, polymorphonuclears early; later small lymphocytes (usually).

10. In syphilis (paresis, tabes, etc.), small lymphocytes predominate.

11. In meningismus (serous meningitis or acute meningeal congestion), endothelial cells predominate.   ✦

QUALITATIVE DETECTION OF PROTEIN

Principles.—1. Normally the spinal fluid contains from 15 to 40 milligrams of protein per 100 c.c., with an average of about 25 milligrams or 0.025 per cent. This is largely in the form of serum globulin. Slight differences occur according to the location or level from which fluid is taken.

2. A large number of tests have been devised for the detection of an increase of protein in spinal fluid. Most of these have been for the detection of the globulins, but practically all react to some extent to serum albumin. Some were originally considered specific for syphilis of the central nervous system but none are pathognomonic for syphilis or any other disease; they merely detect an increase of protein (mostly·globulins) which is always pathological unless the cerebrospinal fluid contains sufficient blood to yield positive reactions.

3. None of these tests, therefore, are applicable to cerebrospinal fluids containing macroscopic amounts of blood. Heavy bacterial contamination may likewise yield falsely positive reactions.

Pandy's Test.—1. Place about 1 c.c. of phenol reagent in a test tube.

For preparing the *reagent,* place 100 c.c. of pure carbolic acid (melt crystals by standing bottle in hot water) in a bottle and add water up to 1000 c.c. Shake vigorously and stand in incubator for several days. Carefully pipet off supernatant fluid or use it direct from the bottle without disturbing the layer of acid.

2. Add 1 drop of fluid to be tested.

3. If there is an increased amount of protein, a bluish white ring or cloud is immediately formed.

Noguchi's Test.—1. In a small test tube place 0.2 c.c. of the fluid to be tested.

2. Add 1 c.c. of a 10 per cent solution of butyric acid in normal salt solution.

3. Heat to boiling.

4. Add 0.2 c.c. of normal sodium hydroxide.

5. Allow to stand for five to ten minutes.

6. A faint opalescence is normal; a precipitate indicates a positive reaction.

7. This test is very sensitive but objectionable because of the disagreeable odor of butyric acid and because a faint but definite opalescence is normal and may be mistaken for a positive reaction.

Ross-Jones' Test.—1. Place 1 c.c. of saturated ammonium sulphate reagent in a test tube.

The *reagent* is prepared by placing 85 grams of Merck's purified and neutral ammonium sulphate and 100 c.c. of distilled water in an Erlenmeyer flask; heat to boiling until all of the salt is dissolved. Cool slowly and filter.

2. Overlay with 1 c.c. of the fluid to be tested.

3. The appearance of a turbid ring at the point of contact indicates a positive reaction. Normally no ring at all is formed at the end of fifteen minutes.

Nonne-Apelt's Test.—PHASE 1 FOR GLOBULIN.—In a small test tube place 2 c.c. of spinal fluid and 2 c.c. of the ammonium sulphate reagent (see above). Allow to stand three minutes. Compare with spinal fluid. A normal fluid gives no reaction or but a faint opalescence.

PHASE 2 FOR ALBUMIN.—Filter contents of tube just prepared; acidulate with acetic acid and boil. A normal fluid remains clear or but faintly opalescent.

Meyerhofer Reduction Test.—This test is an approximate measure of the total organic matter of spinal fluid but does not possess any more diagnostic value than the simple protein tests given above. It depends upon the principle of the reduction of potassium permanganate by spinal fluid in an acid medium.

1. To 1 c.c. of cerebrospinal fluid add 50 c.c. of distilled water, 10 c.c. of 1 : 4 sulphuric acid and 10 c.c. of N/10 potassium permanganate solution.

2. Boil for ten minutes.

3. Add 10 c.c. of N/10 oxalic acid.

4. Titrate with N/10 potassium permanganate until the characteristic color returns.

5. Normally, 2 c.c. or less of permanganate solution are required (normal index). From 2 to 2.5 c.c. are regarded as border line and above 2.5 c.c. as pathological.

QUANTITATIVE ESTIMATION OF TOTAL PROTEIN

Principle.—Proteins are precipitated in finely divided state by sulphosalicylic acid and the turbidity is colorimetrically compared with that of a similarly treated standard protein solution prepared from blood serum.

Procedure (Dennis and Ayer).—1. Into a small test tube pipet 0.6 c.c. of spinal fluid.

2. Add with pipet 0.4 c.c. of distilled water.

3. For the standard, pipet into a similar tube 3 c.c. of standard protein solution.

The *standard* is prepared as follows: (a) Procure about 25 c.c. of fresh normal human blood serum.

(b) Dilute 25 c.c. of this serum to 250 c.c. in a volumetric flask with 15 per cent solution of sodium chloride. Filter. This is the concentrated stock standard.

(c) Determine the total nitrogen of the above filtrate by macro-Kjeldahl method (Kjeldahl-Gunning). A 25 c.c. sample will be convenient.

(d) Determine the nonprotein nitrogen of the original undiluted serum by the micro method of Folin-Wu. Duplicate determinations should be run.

(e) Subtract one-tenth of the serum nonprotein nitrogen value from the total nitrogen of the concentrated stock standard and multiply the result by 6.25 to get the protein content of the concentrated stock standard.

(f) Dilute the concentrated stock standard with distilled water to make a dilute stock standard containing 30 milligrams of protein per 100 c.c. The following formula may be used:

$$\frac{30 \times \text{c.c. of dilute stock standard desired}}{\text{protein conc. in mg./100 c.c. of conc. stock standard}} = \text{c.c. of concentrated stock standard required}$$

(g) To preserve the standards, add a few crystals of thymol and keep on ice. The concentrated standard is said to keep more than six months and the dilute standard more than twelve months without appreciable change.

4. To the unknown add 1 c.c. and to the standard add 3 c.c. of 5 per cent sulphosalicylic acid solution.

5. Mix by inversion (but not by violent shaking) and after standing five minutes compare in a colorimeter, using small cups.

6. Calculation:

$$\frac{S}{R}\times 30 + \frac{1}{0.6} \text{ or } \frac{S}{R}\times 50 = \text{milligrams total protein per 100 c.c.}$$

7. While 0.6 c.c. of spinal fluid is found most convenient for use with normal or approximately normal fluids, it will frequently be found too great in fluids with an increased protein content. With such material it is frequently necessary to use 0.3, 0.2 or even 0.1 c.c. of fluid and to add 0.7, 0.8, or 0.9 c.c. of water respectively (in order to bring the volume of the diluted fluid to 1 c.c.). With some fluids of extremely high protein content, dilution 1:1 with water may be necessary as even 0.1 c.c. fluid may contain too much protein to read against the standard.

8. It is absolutely essential that the standard be placed in both cups and the light and mirror be adjusted so that light values are equal on the two sides.

9. Three factors influence the accuracy of the test and may render it worthless: (1) A fluid contaminated with enough blood to be visible to the eye will in normal fluids give such high protein readings as to be definitely abnormal. (2) Fluids with bacterial contamination will give unreliable results. (3) Fluids standing for long periods uncorked or with cotton plugs, even though clear, will give increasing amounts of protein from day to day. If kept corked and sterile, accurate determinations can be obtained at intervals over a number of weeks.

10. When carefully carried out the method is accurate to approximately 5 per cent.

11. Normal values for lumbar spinal fluid probably lie between 15 and 40 milligrams per 100 c.c. but there are probably some normal as high as 60.

Sicard-Cantelouble Method.—This method employs a glass tube of 7 millimeters inside diameter, 19 centimeters long, graduated in c.c. to 4 c.c., the lower 2 c.c. being further graduated in 0.2 c.c. The method is as follows:

Place 4 c.c. spinal fluid in the tube, heat to 60° to 80° C., and add twelve drops of 33⅓ per cent trichloracetic acid. After five minutes invert a few times. Let stand twenty-four hours, and read quantity of sediment precipitation:

1st graduation...........	0.22 gram protein per liter
2nd graduation...........	0.40 gram protein per liter
3rd graduation...........	0.56 gram protein per liter
4th graduation...........	0.71 gram protein per liter
5th graduation...........	0.85 gram protein per liter

The normal does not exceed 0.30 gram per liter.

Exton's Method.—This is conducted in the same manner as described in Chapter VI for the quantitative determination of albumin in urine, the results being read in milligrams per 100 c.c. from the scale of the Junior Scopometer.

QUALITATIVE DETECTION OF SUGAR

Principle.—1. Normally the cerebrospinal fluid contains from 0.055 to 0.065 per cent of sugar (dextrose) which may be absent in acute suppurative meningitis and reduced in chronic meningitis (tuberculous and syphilitic).

2. Ventricular fluids are likely to contain slightly more sugar than lumbar fluids.

3. Since spinal fluid sugar varies according to the blood sugar, the fluid should be drawn after fasting overnight and compared with coincident blood sugar determinations when *quantitative* determinations are to be made.

4. Qualitative test with the Benedict reagent is essentially similar to the tests for sugar in the urine except that the reagent is diluted and slightly larger amounts of fluid are employed.

5. If the fluid being tested contains an increase of protein and an absence of sugar, the color of the reagent may be changed to a deep purplish-violet or pinkish-violet (the biuret reaction with copper).

6. Since blood sugar will give positive reactions, spinal fluids containing macroscopic amounts of blood are unfit for testing.

Procedure.—1. In a test tube place 0 5 c.c. of Benedict's *qualitative* reagent and add about 4.5 c.c. of distilled water.

2. Heat to boiling and add 1 c.c. of cerebrospinal fluid.

3. Boil for one to two minutes and allow to cool.

4. A change of color to turbid greenish-yellow is a normal reaction for the normal sugar of spinal fluid. No color change shows an absence of sugar and is pathological. An excess of protein may give a biuret reaction as mentioned above.

QUANTITATIVE DETERMINATION OF SUGAR

Principle.—Any proteins present are precipitated by tungstic acid and determination is carried out by the Folin-Wu method, using a 1 : 5 dilution of filtrate.

Procedure.—1. With a 1 c.c. pipet transfer 1 c.c. of cerebrospinal fluid to a clean, dry test tube.

2. Add with a pipet 3 c.c. distilled water.

3. Using a graduated 1 c.c. pipet, add 0.5 c c. 10 per cent sodium tungstate.

4. Add 0.5 c.c. of two-thirds normal sulphuric acid.

5. Mix well and let stand five to ten minutes.

6. Filter.

7 Pipet 2 c.c. of the clear filtrate into a Folin-Wu sugar tube and proceed as with blood.

8. Calculation, using standard 1:

$$\frac{20}{R} \times 50 = \text{milligrams dextrose per 100 c.c. fluid}$$

9. Pipet and test tubes should be absolutely clean and dry or error will be large.

10. If the cerebrospinal fluid contains a little blood, centrifuge and use the clear supernatant fluid.

QUANTITATIVE ESTIMATION OF CHLORIDES

Principles.—1. Normally the cerebrospinal fluid contains from 720 to 750 milligrams of chlorides (expressed as sodium chloride) per 100 c.c., according to the method of Van Slyke, as compared with 570 to 620 milligrams per 100 c.c. of blood plasma.

2. The chloride content of lumbar, cisternal and ventricular fluids is essentially the same.

3. The chloride estimation is valuable in diagnosis when considered in conjunction with the quantity of sugar present. Chloride values below 600 milligrams are infrequent except in tuberculous meningitis, while values between 630 and 680 milligrams per 100 c.c. are commonly found in acute purulent meningitis.

4. Since the presence of blood in spinal fluid increases the chloride content, quantitative determinations cannot be done on fluids containing macroscopic amounts of blood.

5. Qualitative tests are useless.

Procedure.—Same as for the qualitative estimation of plasma chlorides described in Chapter V.

LANGE COLLOIDAL GOLD TEST

Principles.—1. Normal cerebrospinal fluid does not visibly precipitate gold in colloidal suspension in properly prepared Lange's reagent.

2. In syphilis of the central nervous system precipitation may occur, varying from a slight precipitation indicated by a change of color from orange-red to reddish-blue to purple-blue to pale blue to complete decolorization. By means of these color changes, curves of precipitation may be plotted which have a great deal of diagnostic value. Less characteristic precipitations (color changes) occur in tuberculous and acute suppurative meningitis and may also occur in acute anterior poliomyelitis and other diseases of the central nervous system.

3. The exact chemical and immunological nature of the precipitating substance in spinal fluid is unknown.

4. While the test is simple and easily set up and read, the reagent is difficult to prepare and, unless just right with the proper color and neither too sensitive nor too resistant (protected), may readily yield falsely positive or falsely negative reactions. The colloidal gold reaction is, therefore, a valuable diagnostic test but greatly subject to technical errors.

5. The reagent is not very stable and it is recommended that the test

should not be done at all (but the mastic or benzoin tests conducted) unless the worker knows how to prepare and standardize the reagent.

Preparation of Reagent (Modified by Miller, Brush, Hammers and Felton).—1. The preparation of all glassware (beakers, flasks, bottles for reagents, pipets and stirring rods) is very important. Beakers and flasks should be of Jena, Nonsol or Pyrex glass. All glassware should be boiled or well washed with Ivory soap and water, rinsed with running tap water, soaked for thirty minutes or longer in hot bichromate cleaning fluid and *immediately before use* rinsed thoroughly in running tap water, distilled water, and lastly in triply distilled water. The thermometer should be carefully washed and rinsed with distilled water just before use.

2. The water used for preparing the reagents and the colloidal gold reagent should be triply distilled with avoidance of rubber connections in the still. It is recommended that the third distillation be done just before preparing the reagent.

3. The following reagents are required: (*a*) A 1 per cent solution of Merck's yellow crystals of gold chloride in triple distilled water; (*b*) a 2 per cent solution of Merck's blue label potassium carbonate in triple distilled water; (*c*) a 1 per cent solution of Merck's blue label oxalic acid in triple distilled water; (*d*) Merck's highest purity formalin (40 per cent formaldehyde in distilled water).

4. Place 1000 c.c. of triply distilled water and a thermometer in a prepared beaker and heat slowly with a triple Bunsen burner.

5. At 60° C. add 10 c.c. of the 1 per cent gold solution and 7 c.c. of the freshly prepared 2 per cent potassium carbonate solution, using the thermometer as a stirring rod.

6. At 80° C. add slowly 10 drops of the 1 per cent solution of oxalic acid while briskly stirring.

7. At 90° C. turn out the flame and add the formalin drop by drop while constantly stirring until the *first pink color appears* (approximately 5 c.c. ordinarily required).

8. The reagent should gradually assume a brilliant orange-red color without bluish tint.

9. A *shorter method* usually yielding satisfactory reagents is as follows: (*a*) Into a beaker cleaned as outlined above, place 500 c.c. of singly distilled water. (*b*) Place beaker on asbestos-filled wire gauze and heat over Bunsen burner. Stir the solution to keep the heat distributed while heating and gradually add 5 c.c. of 1 per cent solution of gold chloride. (*c*) As soon as the solution begins to boil, turn off the flame and add 4 c.c. of 2 per cent potassium carbonate solution and 0.15 to 0.2 c.c. of 0.05 per cent solution of tannic acid in distilled water. (*d*) Allow solution to come to boil and add 5 c.c. of a 1 per cent solution of oxalic acid while stirring. Remove from flame. (*e*) The solution should develop a salmon or orange-red color and be perfectly clear.

10. *Before use the reagent must be neutral.* (*a*) Place 5 c.c. in a shallow evaporating dish. (*b*) Add 2 drops of a 1 per cent solution of alizarin red in 50 per cent alcohol (indicator). (*c*) A neutral reaction is indicated by a brownish-red color. (*d*) An acid reaction is indicated by a lemon-yellow color. Titrate with N/50 sodium hydroxide solution and calculate amount of N/1 or N/10 sodium hydroxide required for the neutralization of the balance of the reagent. (*e*) An alkaline reaction is indicated by a reddish-purple color. Titrate with N/50 hydrochloric acid and calculate amount of N/1 or N/10 hydrochloric acid required for the neutralization of the balance of the reagent.

11. The finished reagent should fulfill the following requirements: (*a*) Be neutral to alizarin red; (*b*) be crystal clear and of a brilliant salmon or orange-red color with no trace of blue; (*c*) be completely precipitated (rendered colorless) in one hour by the addition of 1.7 c.c. of a 1 per cent solution of sodium chloride in distilled water; (*d*) give a typical paretic curve reaction (like 5554321000) when tested with a known paretic spinal fluid; (*e*) give no more than a 1000000000 reaction with a known normal spinal fluid.

Procedure.—1. Place eleven *chemically clean* test tubes in a rack.

2. Into the first tube place 1.8 c.c. of *0.4 per cent sodium chloride solution* and 1 c.c. in each of the remaining ten tubes.

3. Add 0.2 c.c. of spinal fluid to the first tube and thoroughly mix.

4. Remove 1 c.c. from the first tube and place in the second tube; mix thoroughly and remove 1 c.c. and place in the third tube; continue until the tenth tube is reached and then discard 1 c.c. from this tube. The eleventh tube is used as a control.

5. Add to each tube 5 c.c. of colloidal gold reagent.

6. Mix thoroughly and set aside for twenty-four hours, at the end of which time the readings are made.

7. Readings: Each tube is examined and the reaction recorded, using the numbers 0 to 5.

> 0 = unchanged as compared with the control
> 1 = reddish-blue
> 2 = lilac or purple
> 3 = blue
> 4 = almost colorless (trace of blue)
> 5 = colorless

8. The readings are recorded in the order in which the tubes stand. For example:

5, 5, 5, 5, 4, 3, 1, 0, 0, 0 = curve in the paretic zone (Zone I) as shown in Plate VII
1, 1, 2, 3, 2, 1, 0, 0, 0, 0 = curve in the luetic zone (Zone II) as shown in Plate VIII
0, 0, 1, 1, 1, 2, 2, 3, 2, 1 = curve in the meningitic zone

The chart shown in Figure 152 is recommended for reporting the results of the colloidal gold and other spinal fluid examinations.

CEREBROSPINAL FLUID EXAMINATION

NAME:
CLINICAL DIAGNOSIS:
AGE:
PHYSICIAN:
DATE:

PRESSURE (MILLIMETERS OF MERCURY)	AMOUNT REMOVED (C.C.)	PHYSICAL PROPERTIES	CELLS PER C. MM.	DIFFERENTIAL CELL COUNT			PROTEIN TESTS					COMPLEMENT-FIXATION REACTION	
							QUALITATIVE	QUANTITATIVE				BLOOD	SPINAL FLUID
				LYMPH.	POLYS.	ENDOTHEL.	PANDY	0.2	0.1	0.05	0.025		
Before.													
After.													

COLLOIDAL GOLD AND MASTIC REACTIONS*

REGISTRY NO.	COLOR REACTIONS	DILUTIONS OF SPINAL FLUID										REMARKS
		1 1:10	2 1:20	3 1:40	4 1:80	5 1:160	6 1:320	7 1:640	8 1:1280	9 1:2560	10 1:5120	
5	Colorless..........											
4	Pale blue..........											
3	Blue..............											
2	Lilac or purple.....											
1	Red-blue..........											
0	Red-unchanged.....											

*_____ gold curve; ......mastic curve.
Smears:
Dextrose:
Quantitative Kolmer Reaction:
 Serum 0.1 c.c.
 Serum 0.05 c.c.
 Serum 0.025 c.c.
 Serum 0.005 c.c.
 Serum 0.0025 c.c.

Bacteriological Examination:
Culture:

Animal Inoculation:
Quantitative Chlorids:
Quantitative Kolmer Reaction:
 Spinal Fluid 0.5   c.c.
 Spinal Fluid 0.25 c.c.
 Spinal Fluid 0.125 c.c.
 Spinal Fluid 0.0625 c.c.
 Spinal Fluid 0.03125 c.c.

EXAMINED BY:

FIG. 152.—THE KOLMER CHART FOR SPINAL FLUID EXAMINATIONS.

(From Kolmer, _Chemotherapy_, W. B. Saunders Co.)

CUTTING'S COLLOIDAL MASTIC TEST

Principles.—1. This test depends upon the precipitation of mastic in colloidal suspension as determined by a clarification of the reagent and the production of precipitates.

2. It is highly probable that the substance in spinal fluid producing the reaction is the same as that producing the colloidal gold reaction, although its nature is unknown.

3. The reagent is much simpler and easier to prepare, although the reactions are less sensitive than the colloidal gold reaction.

4. The reaction, while less sensitive, is also less subject to technical errors.

Preparation of Reagents.—1. A stock solution of mastic is prepared by
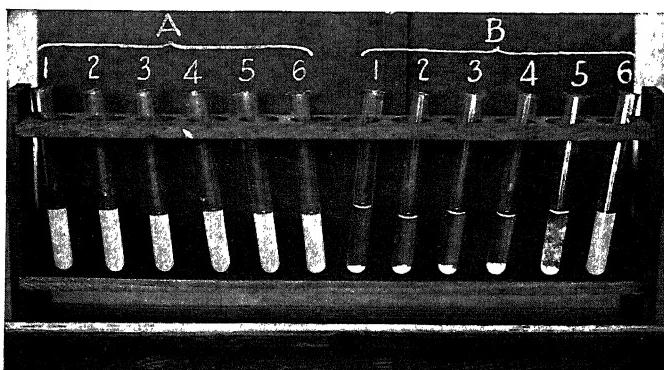
FIG. 153.—COLLOIDAL MASTIC REACTIONS.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

completely dissolving 10 grams of U.S.P. gum mastic in 100 c.c. of absolute alcohol. Filter.

2. For use dilute 2 c.c. with 18 c.c. of absolute alcohol, mix well, and pour rapidly into 80 c.c. of freshly distilled water.

3. Prepare a 1.25 per cent solution of C.P. sodium chloride in distilled water, and to each 99 c.c. add 1 c.c. of a 0.5 per cent solution of potassium carbonate in distilled water (alkaline-saline solution).

Procedure.—1. Arrange six small test tubes in rack.

2. Place 1.5 c.c. of alkaline-saline solution in the first tube and 1 c.c. in each of the remaining five tubes.

3. Add 0.5 c.c. of spinal fluid to the first tube, mix thoroughly and transfer 1 c.c. to the second tube.

4. Transfer 1 c.c. from the second tube to the third and so on until the fifth tube, from which 1 c.c. is discarded. The sixth tube is used as a control.

5. To each tube add 1 c.c. of mastic reagent, mix well and allow to remain

at room temperature for twelve to twenty-four hours; or in the incubator for six to twelve hours.

6. A positive reaction is indicated by the formation of a heavy precipitate which settles, leaving the supernatant fluid clear (Fig. 153).

COLLOIDAL BENZOIN TEST

This test, devised by Guillain, Laroche, and Lechelle, is similar in many respects to the mastic test. It is not specific for neurosyphilis, but gives practically the same results as the more complicated colloidal gold test.

Preparation of Reagents.—1. BENZOIN SOLUTION.—Sumatra benzoin resin, 1 gram; absolute alcohol, 10 c.c. After forty-eight hours filter off the clear supernatant fluid. Keep in a tightly stoppered bottle. This is a stock solution from which the colloidal solution which is used in the test is freshly prepared each day as follows:

Add 0.3 c.c. of the stock benzoin solution, drop by drop with constant shaking, to 20 c.c. of doubly distilled water. Heat to 35° C. in a water bath with constant shaking.

2. SALT SOLUTION.—Make 0.01 per cent sodium chloride in doubly distilled water.

Procedure.—1. Set up in a rack sixteen small test tubes (75 by 10 millimeters, or 85 by 13 millimeters).

2. In the first tube place 0.25 c.c. of salt solution; in the second tube, 0.5 c.c.; in the third, 1.5 c.c., and in each of the remaining tubes 1 c.c.

3. Next add cerebrospinal fluid: 0.75 c.c. to the first tube; 0.5 c.c. to the second and third tubes. From the third tube 1 c.c. of the thoroughly mixed dilution of spinal fluid is transferred to the fourth tube, and so on, until the fifteenth tube is reached, from which, after mixing, 1 c.c. is discarded. The sixteenth tube is used for control. The dilutions thus range from 3 : 4 in the first tube to 1 : 16,384 in the fifteenth tube.

4. Finally, add 1 c.c. of the benzoin suspension to each tube and mix by shaking. The tubes are allowed to stand for from eighteen to twenty-four hours.

5. The reaction will vary from no change in the mixture to complete precipitation of the benzoin, with absolute clearing of the supernatant fluid. The degree of reaction in each tube is reported: 0, no precipitation; 1, slight precipitation, with partial clearing; 2, more than half precipitated, fluid still cloudy; 3, complete precipitation, water-clear fluid. A curve may be plotted, or the figures representing the degree of reaction may be set down for each tube. Precipitation in the first six tubes indicates cerebral involvement, the first, or paretic zone; precipitation beginning with the seventh tube indicates involvement of the meninges, or spinal cord, the second, or meningeal zone. The test is not as sensitive as in the Lange colloidal gold method, and is not so definite in its reaction in multiple sclerosis.

WASSERMANN REACTION

The method of obtaining this reaction is described in Chapter XXVII.

BACTERIOLOGICAL EXAMINATION

1. As soon as spinal fluid is received in the laboratory, it should be cultured on blood agar or some other suitable medium before any other examinations are made, in order to avoid contamination.

2. If very cloudy, direct smears may be made on slides. If opalescent, a portion should be centrifuged and smears prepared of the sediment.

3. Smears should be stained by methylene blue and Gram's method.

4. Methods for the detection and identification of meningococci, pneumococci, streptococci, influenza and tubercle bacilli and other organisms are described in Chapter XIX.

CEREBROSPINAL FLUID IN DISEASE

The accompanying chart briefly summarizes the more important changes in those diseases in which cerebrospinal fluid examinations have proved of value in diagnosis.

SECTION III

BACTERIOLOGICAL METHODS

CHAPTER XV

METHODS FOR THE COLLECTING AND HANDLING OF MATERIAL FOR BACTERIOLOGICAL EXAMINATION

Principles.—*Not infrequently bacteriological examinations are rendered entirely valueless by faulty methods in the collection and handling of material.* The subject therefore is one of considerable importance in which practicing physicians especially require the advice and guidance of bacteriologists. The chief points may be summarized as follows:

1. To obtain the material as free as possible from contamination.

2. As far as is possible *to obtain exactly what it is desired to examine.* For example: In culturing pus from the ethmoid or sphenoid sinuses, a mere swabbing of the nose is not satisfactory if pus may be obtained direct from the infested areas by a rhinologist.

3. *To choose the proper culture medium* if cultures are to be made. For example: If streptococcus or pneumococcus infection is suspected in a chronic otitis media, cultures of pus on plain agar may fail and show only the more rapidly growing organisms, as staphylococci, diphtheroid bacilli, etc.

4. If mixed infection is suspected (as is usually true in chronic infections) to prepare cultures on plates (blood agar recommended) instead of on slants; or to submit the material itself or a swabbing for inoculation of plates in the laboratory.

5. To avoid soiling and contamination of containers, especially in the collection of sputum and feces.

6. To deliver material as quickly as possible to the laboratory after collection.

7. If contamination has occurred or if a defective method of collection has been used influencing the accuracy of the examination, as, for example, submitting smears that are too thin or cultures made on a wrong medium, to report the facts in order to guard against erroneous results and conclusions. For example: A culture of a sore throat on plain agar may show only staphylococci but does not exclude the possibility of diphtheria; or a culture of the eye on plain agar may show nothing but a staphylococcus and fail to grow streptococci or pneumococci if present.

8. With the exception of diphtheria (where the use of Loffler's blood medium is recommended) the most useful routine medium is blood agar in Petri plates. This medium will grow the less hardy organisms, including the hemoglobinophilic group, and thin spreads on plates lessen the chances of slowly growing organisms being overgrown by the hardier, rapidly growing ones.

9. As a general rule sterile swabs are better than platinum wires for securing material, as larger amounts of material are obtained.

10. Material transported to the laboratory should be kept at a low temperature. This minimizes drying, reduces the multiplication of contaminating bacteria and even *B. influenzae* and other delicate organisms can withstand low temperatures for long periods of time. Likewise material received in the laboratory should be placed in a refrigerator until plated or otherwise examined.

COLLECTION OF PUS FROM ABSCESSES AND ULCERS

1. It is preferable to obtain pus at the time an abscess is incised or soon after spontaneous rupture.

2. The surrounding skin should be cleansed with an antiseptic like alcohol, bichloride or mercurophen solution.

3. The pus should be gently expressed and collected on sterile swabs.

4. The infection is usually staphylococcic and cultures may be made on ordinary agar or broth, although blood agar is preferred.

5. Smears are helpful but not required for diagnosis.

6. In suspected *anthrax* of the skin (malignant pustule), smears should be made of the lesion and particularly of the serous contents of vesicles; cultures should be prepared on slants of plain or blood agar.

7. In suspected *tularemia* (ulcers on fingers), smears are useless. Cultures may be made on coagulated egg yolk or blood-glucose-cystin-agar. It is better to inoculate guinea-pigs with material.

8. In suspected *granuloma inguinale,* smears alone are required.

COLLECTION OF MATERIAL FROM THE EYE

1. *It is always advisable to collect the material at a suitable stage of the disease. As a general rule this is during the period in which the disease is developing, or is at its height.* The actual causal agent can disappear rapidly, but the discharge lessens more slowly. In the stage of regression only staphylococci, *B. xerosis,* etc., may be found.

2. In conjunctivitis avoid collecting secretions in contact with the angles or margins of the lids. If it is very scanty, that collected at the inner angle may be used. In dacryocystitis, endeavor to secure fresh material by expression.

3. In corneal infections, *exercise the greatest care* in avoiding injury of

the tissues and spread of the infection. The cornea should be anesthetized and kept perfectly quiet Superficial swabbings may be unsatisfactory. The point of a sterile Graefe knife or needle is sometimes preferred for obtaining material if the real causal organism is likely to be deeply located.

4. Material from the anterior chamber may be aspirated with a small syringe and needle by an ophthalmologist.

5. Portions of iris removed by iridectomy should be placed at once in glucose-brain-hormone broth suitable for the cultivation of streptococci and pneumococci (P^H 7.4 to 7.6).

6. *Smears are always of great value in all eye examinations* as they may show organisms failing to grow in culture media. At least two should be made. Avoid making smears too thin; smears the size of a dime are large enough.

7. In cultures of styes, plain agar may be used because these are caused by staphylococci. In all other infections use only the richer media (blood agar is recommended).

8. Do not make smears or cultures within four hours of the use of a wash or antiseptic solution.

9. Enucleated eyes should be seared or dipped in an antiseptic solution or boiling water for surface disinfection and opened with aseptic precautions for securing portions of the iris, lens, humors and retina for cultures.

COLLECTION OF MATERIAL FROM THE NOSE, SINUSES, AND NASOPHARYNX

1. In culturing the nose, sterile swabs should be used and passed without touching the atrium. They may be first passed above and then below the lower turbinates to the nasopharynx if there are no obstructions.

2. Cultures should not be made within an hour of the application of antiseptics.

3. Secretions may be blown into sterile gauze and portions picked up with sterile swabs (frequently unsatisfactory on account of contamination).

4. Material from infected sinuses should be collected by a rhinologist under direct illumination and with the aid of suction or other special methods for the purpose of securing a small amount of the material direct from the infected areas.

5. Cultures of the nasopharynx should be made through the mouth with curved wire swabs to avoid contamination with saliva. The West tube is useful but not necessary.

6. Plain agar should not be used except for cultures of pus from abscesses which are staphylococcic. Blood agar is recommended for routine use with Löffler's blood serum or hormone broth as second choice since rich media are required for the cultivation of streptococci, pneumococci, *Micrococcus catarrhalis,* meningococci, diphtheria bacilli and such organisms.

COLLECTION OF MATERIAL FROM TONSILS AND FAUCES

1. When inflammatory exudates are present, as in diphtheria, follicular tonsillitis and Vincent's angina, collection with sterile swabs or a sterile platinum loop is sufficient.

2. The swabbing should not be too superficial but an effort made to secure material next to the tissues. This is especially important when diphtheria is suspected, as the bacilli are apt to be deeply located while the surface of a heavy exudate shows nothing but staphylococci. For this reason the first or primary culture may be negative for diphtheria bacilli unless a deep swabbing or a portion of membrane is secured.

3. Löffler's blood serum and blood agar are recommended for the preparation of cultures.

4. Smears on microslides are useful. *In Vincent's angina, smears only are required* as the organisms cannot be cultivated except by very special anaerobic methods.

5. In a bacteriological examination of the tonsils in relation to focal infection, it is advisable and recommended to obtain material from the crypts whenever possible as these are likely to be more satisfactory than surface swabbings. As a general rule these collections are best made by a laryngologist. Material may be expressed from the crypts or secured with the aid of a special sterile glass tube attached to a suction pump. A good method and one that may be conducted in the laboratory is first to make surface swabbings in different directions, as the flora may vary in different locations, followed by a culture of one or more crypts with a platinum loop bent at right angles.

6. Excised tonsils should be delivered in sterile gauze or saline solution immediately after removal. In the laboratory, they may be seared with a hot blade, dipped into boiling water or 70 per cent alcohol for surface disinfection, washed several times with sterile saline and laid open with a sterile knife or scissors. Cryptic material and bits of tissue are then secured and planted in brain-hormone broth or a similar enriched medium adapted for the cultivation of streptococci. Emulsions of tonsil and adenoid tissue may be prepared and cultured.

COLLECTION OF SPUTUM AND BRONCHIAL SECRETIONS

1. Sputum should be collected in a sterile wide-mouthed bottle or vial with the minimum contamination of the mouth and saliva. As a general rule, morning sputum is to be preferred, the patient being instructed to brush the teeth with a boiled toothbrush and to wash the mouth with boiled water before coughing occurs.

2. In suspected *whooping cough*, sputum may be collected in this manner in the case of older children and adults. In young children, faucial secretions may be collected on swabs. Smears are of no value. Cultures should be made on glycerol-potato-blood agar adjusted by acetic acid to P^H 5.0, and plates

of this medium may be held before a child during a paroxysm of coughing for making "droplet" cultures.
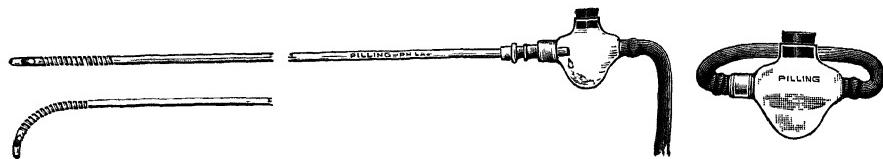
FIG. 154.—TUCKER COLLECTOR.
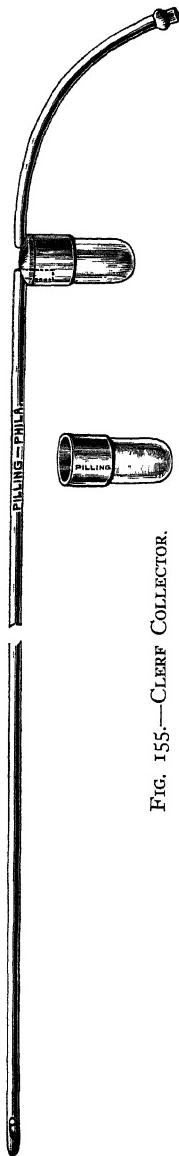
FIG. 155.—CLERF COLLECTOR.

3. Sputum for examination for tubercle bacilli alone by smear methods need not be collected with these precautions. However, when cultures and guinea-pig inoculation tests for tubercle bacilli are to be conducted, they are helpful in reducing the degree of contamination.

4. Sputum to be examined for tubercle bacilli by *smear* alone may be collected in 5 per cent phenol although this is not necessary as the specimens may be autoclaved for sterilization before examination without damage to the morphology or tinctorial properties of the bacilli. In hospitals the collection may be made in paper boxes which are later destroyed by burning, but these cannot be sterilized before examination and are not recommended. Patients should be instructed to carefully avoid contamination of the outside of containers, as the material may be dangerous to handle and bacteriologists are advised not to examine material delivered in such shape.

5. Specimens should be delivered as soon as possible and kept on ice or at a low temperature until examined. Specimens kept twenty-four hours or longer since collection are almost useless for bacteriological examination except for tubercle bacilli by the smear method; older specimens, and especially those kept at room temperature, deteriorate in value for guinea-pig inoculation and culture with increasing time. A method for washing sputum for bacteriological examination is given in Chapter XVIII.

6. Bronchial secretions aspirated with the Jackson bronchoscope are especially well adapted for bacteriological examination in cases of asthma, chronic bronchitis and bronchiectasis. The Tucker (Fig. 154) and Clerf (Fig. 155) collectors are very satisfactory. After use, the collector may be sent to the laboratory, or smears and cultures (blood agar or hormone broth preferred) prepared in the clinic.

COLLECTION OF MATERIAL FROM THE TEETH AND GINGIVAE

1. For the bacteriological examination of *extracted teeth,* the following method is recommended: (*a*) Wall off the operative field with sterile cotton rolls. (*b*) Dry off the area about the tooth with sterile gauze and apply 3 per cent tincture of iodine with care to have it penetrate well into the gingival margin about the tooth. (*c*) Extract, and while holding the tooth in the extraction forceps, sever the apex with a cutting forcep and drop it untouched into a sterile test tube or vial. (*d*) Deliver as soon as possible to the laboratory, where the fragment should be cultured in a rich broth medium like glucose-brain broth; or (*e*) the fragment may be dropped into a sterile screw top vial containing a small amount of sterile sand and gelatin-Locke solution. In the laboratory this should be shaken for ten minutes to macerate the tissue as much as possible and the material transferred with a sterile pipet to tubes of glucose-brain broth and streaked on blood agar plates.

2. Cultures of the *socket* may be made by: (*a*) Walling off the tooth with particular care with sterile cotton rolls to prevent contamination with saliva or the tongue; (*b*) disinfecting the gums with tincture of iodine; (*c*) extracting with forceps with sterilized beaks; (*d*) curetting with sterile curet or with a sterile cotton swab removing material for inoculation in glucose hormone broth or on blood agar plates.

3. Numerous methods have been advised for *culturing the periapical region through the root canals with the tooth in situ* but the following is recommended as a simple one for routine use: (*a*) Isolate the tooth with rubber dam. (*b*) Sterilize the coronal surface with a 3 per cent tincture of iodine. (*c*) Remove the filling with sterile instruments. (*d*) Remove filling or dressing in the root canal with sterile instruments. (*e*) Mechanically cleanse and dry the canal with sterile cotton and insert sterile paper points slightly moistened with sterile saline solution to absorb any moisture oozing into the canal; remove the points and drop them into a tube of glucose hormone broth. (*f*) If no moisture oozes in, pass a sterile, fine broach or pick through the canal and drop it into a tube of the medium.

A more elaborate method recommended by Rickert is as follows: The canals are first opened large enough to be readily accessible. The canal walls should be cleansed with alcohol or hydrogen peroxide. The treatment is introduced on an aseptic cotton point of a length not to exceed two-thirds the length of the canal; above this toward the occlusal orifice place a short section of the dry thickened end of a sterile cotton point; then above this place cotton moistened with sandarac varnish. The cavity is next sealed with either cement or temporary filling. In taking the culture, the tooth and adjacent teeth are isolated, dried and treated with tincture of iodine. The temporary stopping is removed and the cavity moistened with iodine; the sandarac varnish stopping is then removed and this portion cleansed with alcohol. The last dry pledget is then removed with a barbed broach and the dressing to be cultured is carefully

withdrawn; it is seized just above the point of contact of the broach with sterile cotton pliers. The broach, cut off to the pliers and the remaining part of the point which is the apical end, is then introduced into the culture medium.

4. Smears are generally employed for the bacteriological examination of the *gingivae or gums*. Material should be especially collected from the sulci or pockets alongside of the teeth in infection of the periodontium. A stiff platinum loop or some other suitable instrument may be employed; or pus may be picked up with swabs after expression.

Smears may be prepared on microslides in the usual manner, allowed to dry and sent to the laboratory for staining and examination for spirochetes, endamebae, etc. When the secretions are scanty a drop of the patient's saliva or a drop of saline solution should be placed on the slide, the material added, and a smear prepared.

Wet preparations are very useful for examination for spirochetes and endamebae. In this case the patient must be sent to the laboratory or the examination made in the office of the dentist. The spirochetes are readily seen stained or unstained and dark-field examination is not necessary.

Cultures of the surface of the gums are hardly worth while because of inevitable contamination with saliva. But pus expressed from pockets is quite suitable for cultures if the surface is first disinfected with 3 per cent tincture of iodine. The pus is then collected on sterile swabs and sent at once to the laboratory or directly streaked over blood agar plates and then planted in tubes of enriched broth (brain-glucose broth is recommended).

COLLECTION OF MATERIAL FROM THE EAR AND MASTOID

1. In culturing furuncles of the external auditory canal, the skin should be cleansed with alcohol and pus picked up with a small sterile swab. Plain or blood agar or Loffler's blood serum may be inoculated, as these infections are usually staphylococcic.

2. In otitis media the material is best collected by an otologist, as the external auditory canal should be cleansed, disinfected with alcohol and pus obtained on sterile swabs through a speculum and with illumination in order to guard against contamination.

3. In acute otitis media, the organism producing infection is generally obtained in pure culture; in chronic otitis media, two or more organisms are generally found.

4. The pus should be streaked on blood agar plates or inoculated in tubes of enriched broth like glucose-brain broth or glucose-hormone broth. It is a mistake to use plain agar, as this is not suitable for the cultivation of streptococci, pneumococci, etc. In acute otitis, cultures on Löffler's blood serum or slants of blood agar are sufficient, but in chronic otitis, blood agar plates should be used since the infection is generally mixed and rapidly growing

organisms may readily overgrow streptococci and similar slowly growing ones. Smears on slides are also serviceable as their examination gives valuable information, especially in regard to the organisms one may expect to find in the cultures.

5. The same procedures are recommended in mastoid infections. Smears and cultures should be made at the time of operation on blood agar slants or plates or glucose-brain broth suitable for the cultivation of pneumococci and streptococci.

COLLECTION OF CEREBROSPINAL FLUID, PLEURAL AND OTHER TRANSUDATES AND EXUDATES

Cerebrospinal fluid for bacteriological examination should be collected with particular care against contamination, especially if cultures are to be made. The presence of staphylococci in cultures is rather common but of no significance unless skin contamination can be definitely excluded. As meningococci, pneumococci and streptococci in spinal fluid tend to die out rapidly, especially meningococci, *the fluid collected in sterile test tubes should be sent as soon as possible to the laboratory* where large amounts (0.5 to 1.0 c.c.) should be cultured on blood agar, sheep serum agar or similar enriched media. Smears are also of great value and may be prepared after the cultures have been made by smearing the fluid direct if it is purulent or after securing sediment by centrifuging.

Pleural, pericardial, joint and other fluids should be collected by aspiration with a sterile syringe fitted with a sufficiently large needle and under rigid aseptic conditions with particular reference to very careful preparation of the skin. Cultures and smears are then prepared as in the case of cerebrospinal fluid.

COLLECTION OF BILE

1. The technic for collection of bile from the duodenum by nonsurgical drainage is extremely important as the bacteriological examination is almost without value unless the bile is collected with rigid precautions against the several sources of contamination. It is recommended that the method described in Chapter X, employing a special flask, be strictly followed.

2. A broth medium known to be suitable for the cultivation of streptococci should be employed; hormone broth with a P^H of 7.4 to 7.6 is recommended.

3. About 20 drops of bile should be added to 150 c.c. of medium.

4. If a plating method is to be used, bile may be collected in a special sterile vial or test tube.

5. Upon delivery of the specimen, 0.5 to 1.0 c.c. of bile may be removed with a sterile pipet and plated on blood agar, the colonies being examined after twenty-four to forty-eight hours incubation. The broth method, however, is generally more satisfactory.

COLLECTION OF MATERIAL FROM FECES AND THE RECTUM

1. Feces should be passed directly into a quart-size Mason jar previously sterilized by boiling it, the rubber ring and the top for a few minutes before use. Or the patient may pass a stool into a basin previously sterilized by boiling, and a portion (especially feces with mucus) removed with a sterile spatula to a sterile wide-mouthed bottle or vial.

2. Cultures of the rectum for cholera and typhoid carriers may be made by cleansing the skin about the anus with soap, water and alcohol, followed by the introduction of a sterile cotton swab previously moistened with sterile broth or saline solution; or sterile vaselin may be applied to the anus and the finger, covered with a sterile rubber cot, inserted and swabs prepared from the cot. The swabs should be delivered promptly to the laboratory for inoculation of culture media.

3. In ulcerative colitis, cultures are best made by a proctologist with the aid of a speculum and illumination. The ulcers should be first cleansed and material obtained with sterile swabs which should be streaked over blood agar plates or a primary culture made in an enriched broth, like brain-hormone broth for the cultivation of streptococci, etc.

COLLECTION OF URINE

1. Urine to be examined by *culture* should always be collected by catheterization under rigid aseptic conditions.

2. If catheterization is not possible or advisable, especially in infants, the genitalia should be cleansed with soap, water, and alcohol, with special reference to the meatus, and urine collected in a sterile beaker. Special methods and apparatus have been described. It is almost impossible, however, to exclude or prevent contamination, especially with staphylococci and colon bacilli.

3. Urine to be examined for *tubercle bacilli alone by smear* may be collected without any special precautions other than a washing of the genitalia to prevent contamination with smegma. If *guinea-pigs* are to be inoculated, the urine should be passed into bottles previously sterilized by boiling and urine kept at a *low temperature* (especially important during warm weather) to prevent the multiplication of contaminating bacteria and loss of viability of small numbers of tubercle bacilli.

4. Urine to be examined for gonococci, colon bacilli, streptococci, etc., by *smear alone* may be collected in a sterile container after simple cleansing of the parts. It should be centrifuged, however, and smears made within an hour or two after collection, or iced if a longer interval is inevitable, to prevent the multiplication of contaminating organisms.

OBTAINING BLOOD CULTURES

1. Assemble the following:

> 20 c.c. Luer syringe (sterile)
> Sterile needles (20 gauge)
> Soap and water
> Alcohol
> Bichloride of mercury (1 : 1000)
> Tincture of iodine
> Sterile gauze
> Cotton
> Collodion
> Tourniquet
> Alcohol lamp and matches
> Culture media

2. Prepare the patient's arm at the elbow as follows: Wash with soap and water. Apply alcohol over veins at bend of elbow, with sterile gauze. Apply bichloride of mercury (1 : 1000), with sterile gauze. Cover area with sterile gauze saturated with bichloride until ready to take blood (at least one-half hour).

3. *Broth method:* Apply the tourniquet; request the patient to vigorously open and close the hand.

4. Light alcohol lamp.

5. Two flasks of bouillon, 300 c.c. capacity, containing 200 c.c. of glucose-hormone broth (P^H 7.4 to 7.6) should be ready for inoculation.

6. Assemble syringe. Avoid contamination.

7. Remove gauze from arm. Apply tincture of iodine over a prominent vein.

8. Make venous puncture. Avoid touching arm with fingers. If necessary to palpate a vein, cover finger with sterile gauze.

9. Withdraw 10 to 20 c.c. of blood.

10. Release the tourniquet; then withdraw needle from the vein.

11. Direct nurse to attend to arm and immediately remove the needle from the syringe and inoculate the two flasks of bouillon with 5 or 10 c.c. of blood.

12. Place cultures in the incubator at 37° C. for forty-eight hours.

13. Make smears and subcultures if cultures show any evidence of growth. Do not render a report of sterile under ten days' incubation.

14. Contamination with organisms from the skin is not uncommon and should be given consideration. The presence of *Staphylococcus albus* is usually an indication of contamination; even the presence of pigmenting staphylococci should not be considered as coming from the blood of the patient unless confirmed by a second blood culture.

Fox Concentration Method.—1. Fox has recommended collecting 50 to

100 c c. of blood into a sterile 200 c.c. wide-mouthed bottle equipped with a tightly fitting rubber stopper with two perforations   Through each hole passes a glass connection, one going to the needle, the other to a rubber tubing fitted with a glass mouthpiece through which suction is made by the operator to facilitate the flow of blood from the vein.   The blood is collected in the usual way in the bottle containing 100 c.c. of sterile doubly distilled water and 3 grams of sodium citrate.

2. In the laboratory the blood is placed in large tubes and centrifuged for twenty minutes at high speed.

3. Under the greatest precautions the sediment is used for the seeding of culture media (serum-water broth; agar plates; bile broth; glucose broth, etc).

Plating and Counting Method.—1. Melt several tubes of agar (10 c c. each) and place in boiling water in a large vessel with a thermometer.   Cool to temperature between 40° and 45° C.

2. Have patient prepared, and collect blood as just described.

3 Inoculate each tube with 1 or 2 c.c. of blood; mix thoroughly and pour into sterile Petri dishes, taking precautions to prevent contamination.

4. If the bacteria are to be counted, the tubes should contain an accurately measured amount of blood.

5. After incubating plates for forty-eight hours, examine colonies and make smears and subcultures for identification.

6. If count is desired, it can be made by counting the number of colonies on several plates, using a colony counter and calculating the number of bacteria per c.c. of blood.

Citrate Method.—1. In a sterile test tube place 1 c.c. of sterile 10 per cent solution of sodium citrate, and evaporate to dryness.

2. Collect 5 c.c. of blood as described, and place in the tube; rotate the mixture with the citrate to prevent coagulation.

3. In the laboratory, plate 1 c.c. or more of the blood with plain or glucose agar, boiled and cooled to 40° or 45° C.

4. After hardening, incubate the plates, cover down, for forty-eight hours.

5. Count the colonies and estimate number of bacteria per c.c. of blood.

6. Examine colonies for identification.

Method of Cecil and Nicholls for Arthritis Cases.—1. Twenty c.c. of blood are taken aseptically from the arm vein of the patient, and placed in two sterile test tubes.

2. The blood is allowed to clot and placed in the ice box overnight.

3. In the morning the serum is removed and the clots transferred to bottles containing 50 c.c. of beef-heart infusion broth with a P^H 7.6.   The bottles are then placed in the incubator at a temperature of 37° C., and left there for one month.

4. During the month subcultures are made at five-day intervals on blood agar pour plates and in blood broth tubes.   If at the end of thirty days the subcultures remain sterile, the sediment of the original bottle is examined for

organisms by means of smears. Part is streaked on a blood agar plate, and part is transferred to fresh blood broth. If no organism can be demonstrated with this procedure, the blood is considered sterile.

5. All cultures and transfers are made under a hood in order to eliminate contaminations as far as possible. All contaminated cultures are discarded.

COLLECTION OF URETHRAL AND PROSTATIC SECRETIONS

1. Smears of urethral pus are ordinarily sufficient for the bacteriological diagnosis of gonorrhea. But the method of preparing them is of practical importance. At least two smears should be prepared with cotton swabs and rolled (not rubbed) upon slides, care being taken not to pass the swab over the same surface twice. If the amount is scanty, smears the size of dimes are sufficient, as very thin smears are unsatisfactory.

2 It is sometimes advisable to furnish the patient with slides and swabs along with instructions for preparing smears of morning secretions, collected upon arising and before urination.

3. Smears should be allowed to dry in the air. The practice of covering a heavy wet smear with another slide is very unsatisfactory.

4. When cultures are to be made it is advisable to cleanse the meatus and secure pus by urethral massage. This should be picked up with sterile swabs immediately streaked on a suitable medium like that of Pelouze, North, blood agar, etc., or the swabs may be washed out in a small amount of sterile ascites fluid in a test tube furnished with a sterile rubber stopper and at once forwarded to the laboratory for inoculation of media.

5. The examination of urine for gonococci in chronic urethritis is not very satisfactory, although staphylococci, colon and diphtheroid bacilli, etc., from the prostate gland are readily obtained. The prostate gland should be thoroughly massaged and the *first ounce or two of urine immediately passed* collected in one or two sterile centrifuge tubes and used for examination. Or the prostate may be massaged during urination. If cultures are to be made the urine should be secured by catheterization into sterile centrifuge tubes. Whatever method is used it is important to centrifuge the urine as soon as possible at high speed and the sediment should be streaked on a suitable medium and also examined by direct smears.

6. Bacteriological examination of prostatic secretions may be conducted by having the patient empty the bladder immediately before examination. The meatus is then thoroughly washed with soap and water. While the patient constricts the urethra just behind the glans penis, the prostate is thoroughly massaged until fluid collects behind the constriction. This is then collected in one or more sterile Petri dishes and plated on blood agar. Smears may be prepared for the Gram stain at the same time.

COLLECTION OF VAGINAL SECRETIONS

1. Properly prepared smears are still of most value in the bacteriological diagnosis of gonococcus urethritis, vaginitis, bartholinitis, etc., of the female.

2. They should be prepared by rolling swabs of secretion on glass slides (not rubbed on) with care not to pass the swab twice over the same surface.

3. Pus may be secured by massage of the urethra, the Bartholin glands, and from the vagina. In adults with chronic gonorrhea, it is particularly advisable to secure secretions from on and about the cervix with the aid of a vaginal speculum. Vaginal douches should be omitted for at least several hours before examination.

4. Several smears should be prepared and properly labeled. They should be neither too thick nor too thin. If the secretions are scanty, smears the size of pennies are sufficiently large *They should be allowed to dry in the air;* the filthy practice of covering a thick wet smear of vaginal secretion with another slide is strongly condemned

5. Cultures may be prepared by streaking the secretions on plates of ascites agar, North gelatin agar, the Pelouze medium or blood agar. Or swabs may be washed out into small amounts of sterile ascites fluid furnished in small test tubes with sterile rubber stoppers and the ascites emulsion sent at once to the laboratory for the preparation of plates. In medicolegal cases, fresh smears for active spermatozoa and cultures are usually required and the physician should enlist the services of the bacteriologist for aid in the technic of preparing them.

6. In infants and young children smears of the external genitalia are sometimes insufficient and unsatisfactory, especially in chronic infections with scanty secretions.

It is advisable to obtain material from the vagina by means of sterile slender cotton swabs for either smears or cultures or both. In older children, especially in those who have been under treatment, a nasal bivalve speculum may be employed as a vaginal speculum without injury, as it is particularly important to secure secretions on or about the cervix as they may show the presence of gonococci when smears of the external genitalia do not.

Vaginal washings are sometimes serviceable in these cases and may be conducted as follows:

(*a*) Place the child on its back with thighs spread apart.

(*b*) Fill the vagina with 1:4000 bichloride of mercury in normal saline solution by means of a sterile bulb or syringe.

(*c*) Recover the washing and transfer to a centrifuge tube. Repeat until 5 to 10 c.c. of washings have been secured.

(*d*) *Centrifuge as soon as possible* and prepare smears of the sediment to be stained and examined for gonococci.

COLLECTION OF MATERIAL FOR EXAMINATION FOR SPIROCHAETA PALLIDA

1. *Spirochaeta pallida* is best found by dark-field examination of fresh material. Stained smears are much less satisfactory.

2. Wet preparations for dark-field examination may be prepared in the physician's office, providing the microscopy can be done within fifteen minutes and before motility of spirochetes is lost. Otherwise it is better to send the patient to a laboratory equipped for this work.

3. *Spirochaeta pallida* may be found in chancres (genital and extragenital), mucous patches, condylomata and in some skin lesions as well as in swollen lymph glands, although examinations for the organisms are practically confined to sores regarded as possible primary lesions or chancres.

The examination of lesions on the lips is quite reliable but when occurring within the mouth great care is required, since *Spirochaeta microdentium* of the saliva is morphologically indistinguishable from *Spirochaeta pallida*.
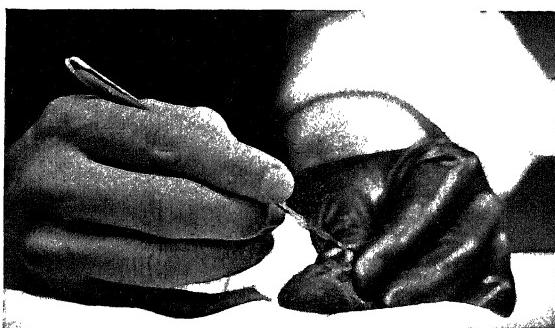
FIG 156 —METHOD OF SECURING CHANCRE MATERIAL.
(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

4. Surface exudates should not be used, as the spirochetes are usually in the tissues. An effort should be made to secure tissue "juice" with as little blood as possible. All applications of antiseptics should be omitted for at least several hours before the examination is made.

5. Thin glass slides (free of scratches) and cover glasses are required; also sterile saline solution and usually an instrument like a scalpel or stiff platinum wire loop for securing tissue juice. Capillary pipets may be used for collection. At least two slides should be prepared. Place a drop of saline solution on each.

6. The lesion should be grasped between the thumb and forefinger (protected with rubber gloves or gauze) and squeezed (Fig. 156) to secure tissue juice which may be transferred to the saline on the slides with a sterilized platinum wire or loop. If this is not successful, the lesion may be gently scraped (while being squeezed to prevent bleeding), the material transferred

to slides and mixed with the saline solution   A cover glass should be applied (being careful to avoid floating) and the dark-field examination made at once. Or smears may be made and allowed to dry in the air if a staining method (like that of Fontana) is to be employed.   If the sore is quite painful, one may first apply a few crystals of cocaine or a few drops of novocaine solution to anesthetize it.

7. Lymph gland material may be obtained by puncture, using a sterile 1 c.c. syringe fitted with a No. 22 needle and injecting 0.5 to 1.0 c c. of sterile saline solution followed by aspiration and the preparation of slides with a few drops of the fluid.

COLLECTION OF MATERIAL FOR EXAMINATION FOR B. DUCREY (CHANCROID)

1. Pus may be obtained by aspiration of a bubo with syringe and needle and inoculated into a medium of two parts agar mixed with one part of sterile human, dog, or rabbit blood; smears should be prepared at the same time.

2. An open ulcer may be painted with tincture of iodine and covered with sterile gauze; twenty-four hours later prepare smears and cultures of pus collecting under the dressing.

COLLECTION OF MATERIAL FOR EXAMINATION FOR LEPROSY

FIG. 157.—SECURING MATERIAL FROM A LEPROUS LESION.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore)

1. Lepra bacilli are usually present in the tissues of leprous lesions in large numbers, especially in tubercular leprosy.   They may also be found in the nose and fauces.

2. The bacilli are chiefly in the fixed tissue cells, and it is usually necessary to scrape the lesions for proper material; little or no pain is produced (Fig. 157).

3. Place a drop of saline solution on each of several slides. Grasp the lesion and, while squeezing to prevent bleeding, scrape with a scalpel or a safety-razor blade and transfer the scraped-up tissue to the slides Make spreads and allow them to dry in the air, to be followed by staining for acid-fast bacilli.

4. Secure material from the nose and fauces with sterile cotton swabs and prepare smears in the usual manner.

COLLECTION OF MATERIAL FROM WOUNDS

1. Smears and cultures are required. It is recommended that purulent secretions be collected in sterile tubes with the aid of sterile swabs and sent to the laboratory. If gangrene infection is suspected, a statement to this effect should accompany the specimen so that special anaerobic methods may be employed for *B. welchii, Vibrion septique,* etc.

2. If cultures are made at the bedside, blood agar plates are recommended, especially if mixed infection is suspected Otherwise blood agar slants on glucose-hormone broth may be used, suitable for the cultivation of streptococci. Plain agar may be used only in case of simple abscesses.

3. If infected wounds are being irrigated according to the Carrel methods, it is necessary to interrupt irrigation for at least two hours. Smears are made on glass slides with a stiff platinum loop or sterile cotton swabs. The areas should be selected with great care with particular reference to the deepest parts, necrosed points of fascia, the surface of damaged bone and culdesacs where secretions can accumulate protected from the irrigation solution. Bleeding areas should be avoided.

4. The secretions thus collected are spread out on slides which should be properly labeled with the name of the patient and the region of the wound whence the secretion was taken. They should be allowed to dry before delivery to the laboratory.

COLLECTION OF NECROPSY MATERIAL

1. When bacteriological examinations are to be made in the course of a necropsy, it is imperative to remove the material at the earliest possible time after death and at least within an hour or two in order to avoid the increase of secondary invaders and the postmortem invasion of the tissues with intestinal bacteria.

2. The body should not be embalmed if cultures are to be made, although smears and examinations for bacteria in sections of tissues may be made.

3. An area of the heart, liver or other organ or tissue to be cultured should be first seared with a cautery iron and then opened with a cautery or sterile knife and material obtained with a sterile pipet, cotton swab, stiff platinum wire or scalpel. Blocks of tissue may be removed, dipped into boiling water for surface sterilization and then cut into bits under aseptic precautions for cultivation in brain-hormone broth or similar enriched media.

REGIONAL CHOICE OF CULTURE MEDIA FOR PATHOGENIC BACTERIA

While the choice of culture media for the bacteriological examination of material from different regions of the body has been indicated above, a brief summary may be presented herewith because of the great importance of the subject, influencing both the accuracy and practical diagnostic value of bacteriological examinations. It is recommended that when cultures are made by the physician or in the laboratory the following culture media be used or substitutes for these of equal or superior nutrient properties:

(a) Pus from furuncles including anthrax: Plain or blood agar slants.

(b) Pus from carbuncles, cellulitis, and adenitis: Blood agar slants and glucose-hormone broth. If tularemia is suspected use coagulated egg yolk and blood-glucose-cystin agar; inoculate guinea-pigs.

(c) Pus from infected wounds: Blood agar plates; special anaerobic cultures if gangrene is suspected; alkaline infusion agar and mouse inoculation if tetanus is suspected.

(d) Conjunctivae and corneae: Blood agar slants and glucose-hormone or brain broth. If tularemia is suspected, use coagulated egg yolk and blood-glucose-cystin agar.

(e) Nose, sinuses and nasopharynx: Blood agar slants or plates or Löffler's blood serum; also glucose-hormone broth.

(f) Tonsils: Löffler's blood serum for diphtheria bacilli; otherwise blood agar slants or plates and brain-hormone broth.

(g) Sputum: Blood agar plates and glucose-hormone broth; special media for tubercle bacilli; mouse inoculation for pneumococci.

(h) Teeth and gingivae: Brain-hormone broth or glucose broth; blood agar slants or plates.

(i) Ear and mastoid: Blood agar slants or glucose broth for acute infections; blood agar plates for chronic mixed infections.

(j) Cerebrospinal, pleural and other fluids: Blood or sheep serum agar plates and brain-hormone broth.

(k) Bile: Plain or glucose-hormone broth (P^H 7.4 to 7.6).

(l) Feces and rectum: Blood agar plates; special media for typhoid-cholera group.

(m) Urine: Plain agar slants or broth for colon-typhoid group; blood agar or hormone broth for streptococci; special media for tubercle bacilli.

(n) Blood: Plain or glucose broth (P^H 7.6).

(o) Urethra, prostate gland and vagina: Pelouze, North or other special media for gonococci; blood agar slants or plates for secondary organisms (*B. coli*, staphylococci, etc.).

(p) Venereal ulcers and buboes: Special blood agar for Ducrey's bacillus (chancroid); wet preparations or smears alone for *Spirochaeta pallida* (syphilis) as cultures are useless.

DISPOSAL OF MATERIAL SUBMITTED

1. It is a good practice to retain specimens of pus, various secretions, spinal and other fluids, etc., for several days at least in case it is necessary or advisable to repeat the examinations. Cultures should be retained for a week or longer in case confirmatory tests are desired. It is also advisable to retain important smears properly labeled.

2. All specimens containing or likely to contain pathogenic organisms should be placed in a pail or pan and the latter autoclaved at the close of the day. Paper sputum cups should be burned.

3. It is a good practice to add a few c.c. of 5 per cent phenol or tricresol to all discarded cultures (replacing the stoppers), including Petri plates.

4. Pipets used for handling infectious material should be placed in a jar, crock, or pan of water containing phenol or tricresol for disinfection.

5. Boil all instruments and syringes immediately after use.

6. Wash the work table routinely with 10 per cent crude cresol.

CHAPTER XVI

METHODS FOR THE PREPARATION AND STERILIZATION OF GLASSWARE

Principles.—Test tubes, Petri dishes, and flasks should be made of good quality glass in order to (1) withstand repeated steam sterilization at 121° C. (approximately 15 pounds pressure) and hot air at 170° C. with the minimum of decomposition and (2) to contain the smallest amounts of free alkali so that there will be the minimum difference between the initial and final reactions of culture media. Strain-tested Pyrex and Nonsol glass are recommended because of their stability toward distilled water, and low coefficient of expansive and mechanical strength.

SELECTION OF GLASSWARE

1. *Test tubes* should be of thicker walls than are used for chemical work, without lips in order to facilitate plugging and storage, and of such size as to fit in the wire test-tube racks now in common use. Three sizes are usually sufficient: 100 by 13 millimeters for slants and broth, as 5 c.c. in each is sufficient and economical; 200 by 13 millimeters for giving a high column of medium with varying degrees of oxygen tension (low at the bottom and high at the top); 120 by 16 millimeters for carrying to 10 c c or
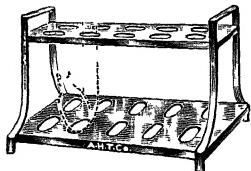
FIG 158—FERMENTATION TUBE SUPPORT

FIG 159 — WIDE MOUTH RECTANGULAR BOTTLE

larger amounts of agar, gelatin, etc , for pouring Petri plates and for holding sterile swabs.

2. *Petri dishes* subjected to steam pressure sterilization should be of alkali-free glass and capable of standing repeated sterilization without corrosion (N–101–AF glass is recommended). Where hot air sterilization only is used, dishes of selected lime glass are sufficient, providing they are thoroughly dried before being placed in the sterilizer. Covers of white Coors porcelain are recommended; unglazed inside for providing sufficient absorbing surface for water of condensation, and glazed outside and at the sides for facilitating the removal of pencil markings. The 10-centimeter size is ordinarily employed.

3. *Erlenmeyer flasks* of Pyrex or Nonsol glass with vial mouths are recommended and three or four sizes are usually sufficient: 300 c.c. capacity for blood cultures and 500 to 2000 c.c. capacities for storage of culture media, etc.

4. *Fermentation tubes* of small size, graduated, and with glass feet, are recommended. If tubes without feet are employed, a support is required (Fig. 158).

5. *Blake bottles* (Fig. 159) and *Kolle flasks* are commonly employed for mass cultures in the preparation of stock vaccines, bacterial antigens, etc.

6. *Microslides* should be of noncorrosive hard glass with grounded edges, free of scratches and of the usual 3 by 1 inch size with a thickness of 1 to 2 millimeters. For dark-field work the slides should be carefully selected, free of scratches and within 1.45 to 1.55 millimeters in thickness.

7. *Micro cover glasses* should be of noncorrosive glass; soft white glass should not be used. Squares (15 millimeters), rectangles (22 by 36 millimeters), and round glasses (15 millimeters) may be recommended for routine work.

CLEANSING GLASSWARE

New Glassware.—New test tubes, pipets, Petri dishes, flasks, microslides and micro cover glasses may be used after simple cleansing with hot water and soap to remove adherent dirt and grease but (with the exception of slides) also require special attention to remove *resistant spores* which are sometimes present in the straw or other packing material. The following method of cleansing is recommended:

1. Boil the glassware for one hour in water to which is added about 50 grams of thin shavings of a good brown soap per liter. Do not use soap powders as they tend to cloud the glass.

2. After cooling, rinse in running water, drain, and dry. Or after rinsing immerse in 1 per cent hydrochloric or nitric acid for an hour to neutralize alkali; rinse *very thoroughly* in running water. A final rinsing in distilled water is advisable but not absolutely necessary. New glassware of the cheaper grades is especially likely to give off free alkali.

3. Slides and cover glasses may be polished ready for work or kept in 70 per cent alcohol and polished as required.

Used Glassware.—Glassware should be washed as soon after use as possible.

Pipets should be placed in a tall crock or cylinder full of 2 per cent lysol solution (for disinfection) with a layer of cotton on the bottom, *immediately after using.* To wash pipets, hold them in flowing tap water, or better, use a water suction pump attached to the faucet. Place the mouth end of the pipet in the rubber tube connected with the pump and the other end in a container of tap water and suck water through for about one-quarter minute; put aside to drain and dry.

Test tubes should be handled with due care against contamination of the

hands, especially with discarded cultures of tubercle, typhoid and diphtheria bacilli, etc.

A good practice is to disinfect each after use by adding a sufficient amount of 2 per cent phenol or lysol solution, or sterilize immediately by boiling.

1. Tubes with paraffined stoppers should be *separately* cleaned because soiling with melted paraffin renders cleaning more difficult. Remove all stoppers and drop them into lysol solution or burn them.

2. Fill and cover the tubes in a bucket with water to which is added 5 per cent soap (not soap powder) and boil for an hour or autoclave at 110° to 120° C. for thirty minutes. In the case of discarded cultures of subtilis, tetanus, anthrax and other spore-forming bacilli, autoclaving is recommended.

3. While still hot, empty and clean each tube with a stiff brush under running tap water. Drain and dry, or place in 1 per cent hydrochloric acid for an hour, *thoroughly* rinse in running water, drain and dry.

Petri dishes may be disinfected immediately after use by covering the medium with lysol or phenol solution, and then boiled or autoclaved and cleansed in the same manner.

Flasks containing living organisms should be autoclaved at
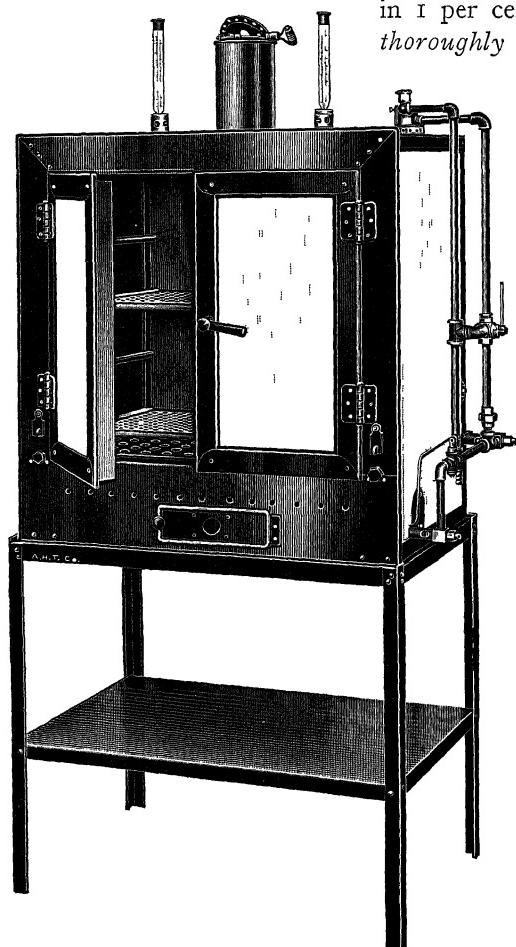
FIG. 160.—HOT AIR STERILIZER.
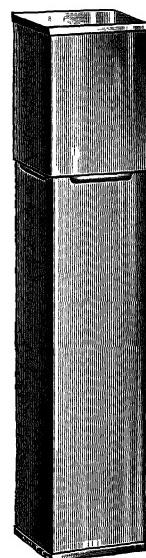
FIG. 161.—PIPET BOX.

110° to 120° C. for thirty minutes before emptying their contents and cleansing in the above manner.

Slides and cover glasses may be placed in 2 per cent lysol solution which in a week or so loosens Canada balsam; this solution also disinfects hanging drop and dark-field preparations.

1. Boil in soapy water or 10 per cent solution of chromic acid for ten minutes.

2. Rinse in running water, drain and polish or keep the slides and cover glasses in 70 per cent alcohol or in a mixture of 2 per cent acetic acid in 70 per cent alcohol until ready for use.

Cloudy Glassware.—When glassware (test tubes, Petri dishes, pipets, slides, cover glasses, etc.) retains cloudiness that cannot be removed by means of washing with soap, use potassium bichromate sulphuric acid cleaning solution, prepared as follows:

> Commercial potassium bichromate .... 120 gm.
> Tap water................. .... 1500 c.c.
> Dissolve with the aid of heat and add:
> Commercial sulphuric acid .... 180 c.c.

Fill test tubes, beakers, and flasks with cleaning solution and place pipets, slides, cover glasses, etc., in pans full of the solution and allow to remain twenty-four hours. To be followed by *thorough* rinsing in running water to remove all traces of cleaning fluid.

The fluid can be used repeatedly and when it appears to lose its strength more potassium bichromate and sulphuric acid should be added. It is, however, very corrosive and should not be used more frequently than necessary.

PLUGGING TEST TUBES AND FLASKS

1. Take two pieces of long-fiber, nonabsorbent cotton (cotton batting), cross them, and with rod push the center of the crossed strands into the tube or flask. The stoppers in test tubes should be large and firm enough to exclude dust and germs, should project sufficiently for handling and be tight enough to permit one to lift the container by means of the plug. They should not be twisted in as this may leave channels for contamination.

2. In the case of flasks requiring large plugs, it is well to place a square of gauze over the mouth of each before pushing in the cotton plug.

3. Added protection against contamination may be given by placing a piece of tin foil over the plug, or a piece of filter paper or unbleached muslin secured with cord around the neck of the flask.

4. It is recommended that a small plug of cotton be placed in the mouth ends of pipets to prevent accidental contamination of the mouth in pipetting broth cultures.

STERILIZING GLASSWARE

1. Test tubes, Petri dishes, pipets and flasks should be *perfectly dry* before hot air sterilization (Fig. 160), to prevent breakage and reduce decomposition. Do not use moist heat.

2. Petri dishes and pipets may be wrapped singly or in multiples with paper. Or pipets may be placed in special cans of copper or sheet iron for sterilization (Fig. 161).

3. Place in hot air sterilizer and *gradually* raise heat to about 170° C., which is sufficient for turning cotton and paper to a faint yellow color.

4. Avoid overheating to prevent charring of cotton and paper and the release of oils from cotton. Do not allow cotton plugs to touch the walls of the oven.

5. Heat for an hour and allow the sterilizer to cool to at least 60° C. before opening the door, to prevent cracking by too sudden contraction of the glass.

6. While it is not imperative to sterilize test tubes and flasks before filling them with culture media, it is advisable to do so for ensuring better sterilization and for the purpose of molding the plugs for easier removal during bacteriological work.

7. When large amounts of glassware are to be sterilized, the use of a good thermo-regulating valve on the sterilizer together with a recording thermometer are recommended.

CHAPTER XVII

METHODS FOR THE PREPARATION OF CULTURE MEDIA

Principles.—1. Culture media are artificial foods for bacteria, containing soluble albumins, carbohydrates and other organic compounds as well as water and salts. Native proteins are probably not directly utilized and peptone is added to supply available nitrogen. Vitamins as well as other special substances help the growth of all pathogenic bacteria, especially streptococci, pneumococci, gonococci and the hemoglobinophilic bacilli like *B. influenzae, B. pertussis,* Ducrey's bacillus, etc., which require "*V*" and "*X*" factors. Certain organisms require the addition of other food substances, such as blood (hemoglobin), serum, ascites fluid, etc. Carbohydrates and especially glucose may be required. Dyes may be added either as indicators of metabolic activity or because of their selective inhibitory action on some bacteria, thereby aiding in isolation of others (notably the colon-typhoid group).

2. Therefore a very large number of media have been described for meeting special requirements. Practically all have as a general basis an infusion or watery extract of meat (usually beef or veal).

3. Heating for sterilization and filtering for clearing affects meat infusion deleteriously, and lost nutritive material is replaced in part by the addition of peptone, which is water-soluble and not precipitated by boiling; also by adding blood, serum, etc. Beef extract is generally inferior to fresh meat infusion because of the loss of nutritive substances due to prolonged heating in manufacture. It is, however, useful for the cultivation of the more hardy organisms and especially convenient for small laboratories.

4. The method of sterilization of culture media is, therefore, an important matter. Autoclaving may destroy nutritive principles. Fractional sterilization in an Arnold sterilizer is generally to be preferred.

5. Most pathogenic bacteria are quite susceptible to acids and alkalis and grow best in media near neutrality or slightly alkaline or acid to it. The meat bases are more or less acid and usually require the addition of an alkali (sodium hydroxide). Culture media tend to become more acid during sterilization because of hydrolysis of some of the constituents. On the other hand, they may become more alkaline because of alkali derived from glass, especially if cheap, soluble glass containers are employed.

6. Culture media may be (*a*) fluid (broth, milk, peptone water, etc.); (*b*) solid (agar, gelatin, coagulated serum or egg, etc.), or (*c*) semisolid

broth with small amounts of agar, gelatin or coagulated serum) ; the last are especially useful for carrying stock cultures.

7. The most important principles involved in their preparation are: (*a*) To secure the maximum amounts of growth-stimulating substances, and (*b*) for this purpose to adjust the final reaction to the optimum P^H for the organisms to be cultivated; (*c*) to use the minimum degree of filtration; (*d*) to use the minimum amount of heat for sterilization.

DETERMINING AND ADJUSTING THE REACTION OF CULTURE MEDIA

Litmus Method.—This is the oldest and simplest method, but is too inaccurate for best results because litmus is not a definite compound and chemically

FIG 162.—OUTFIT FOR PHE-
NOLPHTHALEIN TITRATION.

(From Park, Williams and Krumwiede, *Pathogenic Microörganisms,* Lea and Febiger.)

pure azolitmin (its chief component) varies in its color reactions according to the composition of the medium. It is useful for making preliminary determinations and may be added to culture media for determining the production of acids by bacteria (as in the Hiss serum-water medium for differentiating between streptococci and pneumococci).

For determining the reaction, use red and blue litmus papers. Red to blue=alkalinity; blue to red=acidity. If blue turns red, add sufficient 4 per cent sodium hydroxide solution to give this end-point: red strip to slight blue and blue strip to a shade less blue. Litmus is sufficiently sensitive for ordinary culture media for hardy organisms and the color change occurs at approximately P^H 6.8 to 7.0.

Titrimetric Phenolphthalein Method. — The color changes of phenolphthalein are:

(*a*) Colorless: medium is acid.

(*b*) Faint pink: medium is neutral to phenolphthalein but actually on the alkaline side of true neutrality equal to P^H 8.2 to P^H 10.0.

(*c*) Red: medium is alkaline.

The plus sign is used for denoting acidity and the minus sign for alkalinity, according to Fuller's method.

1. The materials required are (Fig. 162):

(*a*) A buret (B) held in a clamp on a ring stand (A)

(*b*) Casserole (C)

(*c*) Glass stirring rod
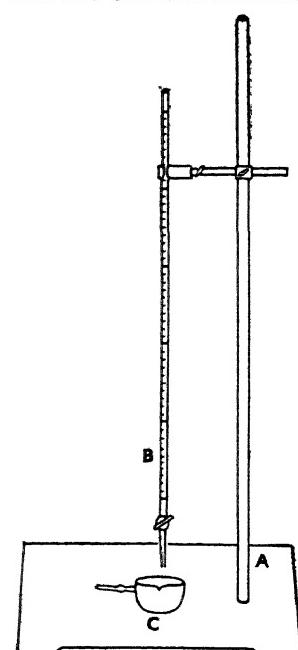
(d) Normal (N/1) and twentieth normal (N/20) sodium hydroxide solutions

(e) Indicator: dissolve 0.5 gram phenolphthalein in 100 c.c. of 50 per cent alcohol (0.5 per cent solution)

2. Put 45 c.c. of *freshly boiled* and cooled distilled water 5 c.c. of medium in the casserole. If agar is being titrated, have the water at about 40° C. to keep the medium fluid.

3. Add 1 c.c. of indicator solution. As a general rule, there is no color change, indicating that the medium is acid.

4. Place N/20 sodium hydroxide solution in the buret and record the reading or level.

5. Add small amounts of the sodium hydroxide solution to the medium, stirring briskly after each addition.

6. Stop at the *first faint pink tinge.*

7. Read the buret and record.

8. Subtract this reading from the first to give the amount of sodium hydroxide required for 5 c.c. of medium. Example: 2.1 c.c. N/20 sodium hydroxide used. The medium has an acid reaction recorded as +2.1 according to the Fuller method.

9. If it is desired to render the bulk of the medium neutral to phenolphthalein, proceed as per the following example:

5 c.c. require 2.1 c.c. of N/20 sodium hydroxide

100 c.c. require 42.0 c.c. of N/20 or 2.1 c.c. of N/1 sodium hydroxide

1000 c.c. require 21 c.c. of N/1 sodium hydroxide

10. A shorter method of calculation is as follows. Let A equal the amount of N/20 required for 5 c.c. of medium and B the bulk of the medium:

$$\frac{A \times B}{100} = \text{c.c. of N/1 sodium hydroxide required for rendering the bulk of medium neutral to phenolphthalein}$$

11. If it is desired to have the medium slightly acid to phenolphthalein, for example, +0.1, add 20 c.c. of N/1 sodium hydroxide instead of 21 c.c. If an end-point of +0.2 is desired, add 19 c.c. of N/1, etc.

12. This method, however, is only approximately correct because the buffer substances present in the medium combine with some of the sodium hydroxide so that one does not know exactly how much acid has been neutralized nor the actual acidity of the medium. For this reason the hydrogen ion method of titration (given below) is to be preferred.

13. Few media are naturally alkaline to phenolphthalein, that is, yield a red color upon the addition of this indicator. But in this event titrate with N/20 solution of hydrochloric acid to determine the quantity required for reducing to neutrality (faint pink color), and calculate as above. If, however, a medium has been rendered too alkaline by the addition of too much sodium

hydroxide, it is better to discard it than to reduce the alkalinity with hydrochloric acid, as this generally renders the medium unsatisfactory for the cultivation of sensitive bacteria.

14. The above is the *room temperature* or *standard method* to be applied to the titration of culture media brought to the boiling point in preparation before titration. If, however, the medium has been heated to only about 50° C. for dissolving the ingredients, it is necessary to boil the mixture of 5 c.c. in 45 c.c. of distilled water for one minute before the titration is conducted (the *boiling method*) to allow for the chemical changes to which the bulk of medium will be subjected during sterilization.

Colorimetric Hydrogen Ion Method.—The hydrogen ion concentration of a solution indicates the weight, in grams, of its ionic hydrogen per liter. Distilled water has a hydrogen ion concentration of 0.000,000,1, which is expressed as 1×10^{-7}. The symbol P^H has been suggested in the place of the logarithm of the reciprocal and is used with the negative exponent for indicating the hydrogen ion concentration of a solution. The concentration of water would be therefore expressed as P^H 7 and this represents the neutrality point in the P^H scale.

In the P^H scale acid reactions scale lower than 7 and alkaline reactions scale above 7.

The P^H of culture media is not increased or decreased in direct proportion to the amount of acid or alkali added. This is due to the action of buffer substances present in the media.

Buffers are substances which tend to inhibit a change in the P^H when acid or alkali is added to a solution. Such substances as peptone, meat extracts, and phosphates have this inhibitory quality. Knowing the P^H of a solution, it is not possible to figure mathematically the exact amount of acid or alkali to add to obtain a certain lower or higher P^H, because of the action of these buffers.

Indicators are substances which when added to a solution assume a definite color at a particular P^H. One indicator is useful only for a certain limited range of the P^H scale. This is from the P^H at which color begins to change to the P^H where the color change is complete, *e.g.,* phenol red gives a yellow color in solutions with low P^H (acid side); at a P^H 6.8 it begins to change to a pale pink which is intensified until a red is assumed at a P^H 8.4 (alkaline side) and above.

It is obvious that several indicators are required to cover the entire P^H range. But for the adjustment of culture media only two or three are required to cover the P^H range usually employed.

Color standards of the indicator can be prepared in standard buffer solutions to represent the shade of color obtained with a particular indicator at various points in the P^H scale. However, these are difficult to prepare as each buffer solution, which is usually a mixture of some acid and its alkali salt, should be very carefully tested by the electrometric method for checking the exact P^H.

Standard phosphate solutions (Sorensen) may be prepared as follows:

1. Prepare a $M/15$ solution of sodium phosphate (Sørensen)[1] in ammonia-free distilled water.

2. Prepare a $M/15$ solution of potassium phosphate (Sørensen)[2] in ammonia-free distilled water.

3. Mix the two solutions in various proportions, measuring with extreme accuracy:[3]

$M/15$ Sodium Phosphate, c.c.		$M/15$ Potassium Phosphate, c.c.		P^H
97 5	+	2 5	=	8.3
95 0	+	5 0	=	8 0
92 0	+	8 0	=	7 8
88 0	+	12 0	=	7 6
82 0	+	18 0	=	7 4
73 0	+	27 0	=	7 2
62 0	+	38 0	=	7 0
50 0	+	50 0	=	6.8
37 0	+	63 0	=	6 6
26.0	+	74 0	=	6 4
18 0	+	82 0	=	6.2
12.0	+	88 0	=	6 0

4. Unless the laboratory is especially equipped for preparing and testing the standard solutions, it is advisable to purchase them. Standard sets covering the range of any of the indicators used in adjusting culture media can be purchased from laboratory supply houses.

The indicators most commonly used for bacteriological work are:

> Bromcresol purple = yellow to purple = P^H 5.2 to 6.8
> Bromthymol blue = yellow to blue = P^H 6.0 to 7.6
> Phenol red = yellow to red = P^H 6.8 to 8.4

The bromcresol purple and bromthymol blue are used in 0.04 per cent and phenol red in 0.02 per cent solutions in 95 per cent alcohol.

The *technic* of the colorimetric method is as follows:

1. Materials required: (*a*) freshly distilled water (ammonia free); (*b*) test tubes; (*c*) comparator block (Fig. 163); (*d*) several 10 c.c. pipets; (*e*) buret; (*f*) standard color tubes of the desired P^H range (Fig. 164).

2. Place 2 c.c. of the medium to be tested and adjusted in each of two test tubes. Add 8 c.c. of freshly distilled water to each. If the water has been exposed to air for some time before use or is not freshly distilled, it should be boiled and allowed to cool to below 40° C. just before use. If agar

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[1,2] These salts should be especially prepared for this purpose. They can be obtained from reliable drug houses under the name of Sørensen's potassium phosphate and sodium phosphate.

[3] These mixtures are not stable and remain good for but two to three weeks.

is to be tested it can be measured while liquid and diluted with warm water (not over 40° C.) to prevent solidification.

3. To one of the tubes containing diluted media, add the same amount of indicator as used in the standard tubes (ordinarily 0.25 or 0.5 c.c.). Place the tube in the right front hole of the comparator block.

4. The other tube of diluted medium is placed in the left front hole of the comparator block.

5. Fill a third tube with distilled water and place in the right back hole of the comparator block, which is therefore behind the tube containing medium plus indicator.

6. From the set of standard color tubes select one which appears to match the tube of medium plus indicator. Place it in the left back hole, which is in back of the tube containing medium only. Compare the colors by viewing through the observation holes of the comparator block with either a daylight lamp or window furnishing light from the back of the block.
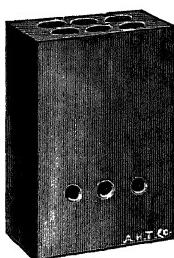
FIG. 163. — CLARK COLOR COMPARATOR.
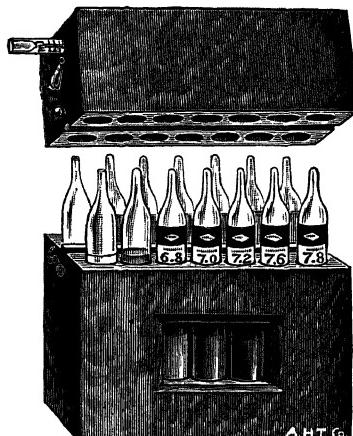
FIG. 164.—LAMOTTE HYDROGEN ION SET.

If the colors do not match, select another standard tube either lighter or more intense in color as may be indicated, and again examine.

Repeat this procedure until the tube is found which matches. The P^H value marked on the tube which matches indicates the P^H of medium.

7. To change the P^H of a medium to another point in the P^H scale, place the standard tube marked with the P^H desired in the left-hand back hole and allow the other three tubes to remain in the same positions.

8. Slowly and carefully add N/20 solution of sodium hydroxide drop by drop to the tube containing medium and indicator until the color matches the color standard tube of the desired P^H. Note the amount of N/20 solution used. The addition should be made from a buret or pipet and carefully measured. It is necessary to mix at intervals during the addition of the

sodium hydroxide, especially when approaching the end-point, in order to avoid adding an excess.

9. To adjust the bulk of medium it is necessary to add to it sodium hydroxide in the same proportion as used to adjust the sample tested.

Suppose there are 2000 c.c. of medium to be adjusted to a P^H 7.6, and that it required 0.3 c.c. of N/20 sodium hydroxide to bring the tube containing 2 c.c. medium and indicator to the same color as the standard tube marked P^H 7.6. It would therefore require 300 c.c. of N/20 sodium hydroxide to adjust 2000 c.c. of medium. But the addition of this amount of N/20 would increase the volume too much, so it is better to add one-twentieth of this amount or 15 c.c. of a N/1 solution of sodium hydroxide.

The following is a simple method for calculation. Let A equal the amount of N/20 required to adjust 2 c.c. of the medium to the proper P^H, and B the number of c.c. in the bulk of medium to be adjusted:

$$\frac{A \times B}{40} = \text{c.c. of N/1 sodium hydroxide to add to bulk of medium}$$

or

$$A \times 25 = \text{c.c. of N/1 sodium hydroxide to add to each liter of medium}$$

Optimum P^H for Various Pathogenic Organisms.—The following P^H reactions of culture media for the commoner pathogenic bacteria are recommended:

	P^H
B. typhosus	6.2 to 7.2
Russel's double sugar	7.4 to 7.6
Endo	7.8 to 8.0
B. paratyphosus A and B	6.2 to 7.2
B. dysenteriae (Shiga)	6.2 to 8.4
B. coli	6.0 to 7.0
B. cholera	6.2 to 9.0
M. melitensis	6.6 to 8.2
Pneumococcus	7.0 to 7.6
Streptococcus	7.6 to 7.8
Meningococcus	7.6 to 7.8
B. influenzae (?)	7.6 to 7.8
Gonococcus	7.3 to 7.6
B. diphtheriae	7.3 to 7.6
B. anthracis	7.0 to 7.4
Staphylococcus	7.0 to 7.6
B. tetani	7.0 to 7.6
B. tuberculosis	6.8 to 7.2

Method of Using Indicators in Culture Media for Showing Changes in Reaction Due to Bacteria.—1. The degree of acidity or alkalinity produced in culture media *after* bacterial growth is best determined by taking 5 c.c. and determining the hydrogen ion concentration by the colorimetric method.

2. For determining a change of reaction *during* bacterial growth, various indicators without antiseptic or bactericidal activity may be added to culture media as follows:

(*a*) Five-tenths c.c. of a sterile 5 per cent solution of litmus to 10 c.c. of medium. Dissolve 5 grams of Merck's or Kahlbaum's litmus in 100 c.c. of water by steaming in an Arnold sterilizer; filter; sterilize in Arnold for one hour. The medium should be neutral or slightly alkaline. The growth of acid-producing bacteria changes the color to a red or reddish shade. The litmus, however, may be decolorized to a leukobase by the growth of bacteria absorbing the oxygen (color returns on exposure to air) and this must not be mistaken for a change in chemical reaction.

(*b*) One per cent alcoholic solution of phenolphthalein may be added to give a faint pink color. Medium may be autoclaved. Color disappears as acid is produced.

(*c*) The newer indicators may be used: bromcresol purple for media with a P^H 5.2 to 6.8; bromthymol blue for P^H 6.0 to 7.6, and phenol red for P^H 6.8 to 8.4. Use sufficient for giving the desired shade (ordinarily 1.5 to 2.0 c.c. of 1.6 per cent alcoholic solutions). These indicators are not reduced by absorbing of oxygen and are not affected by autoclaving.

(*d*) Various aniline dyes may be used as in the Endo and Andrade indicators in agar media for the identification of colon and typhoid bacilli, etc.

CLEARING AND FILTRATION OF CULTURE MEDIA

1. Culture media should be clear, but too fine filtration may remove growth-stimulating substances.

2. Large particles may be removed by sedimentation as in the preparation of "vitamin" media: After heating, allow broth media to stand overnight and decant or pipet off the supernatant fluid next day without disturbing the sediment. Allow agar to stand overnight in a *straight side* container; turn out, trim off and discard the sediment. The finer particles remain in suspension and no further attempt is made at clearing except filtration through a fine wire mesh.

3. Clearing is also accomplished by coagulation of albumins during heating (boiling, Arnold sterilizer or autoclave) with enmeshment of particles, or by adding an egg to each liter of medium to furnish coagulable albumin. Mix one egg in a pan with an equal amount of water and add. If dried egg albumin is used, dissolve 10 grams in 20 c.c. of water and add. The medium is then heated in Arnold sterilizer for forty-five to sixty minutes or autoclaved for thirty minutes; ordinary boiling is not as good. Clearing with egg, however, should be avoided whenever possible because of the possibility of adding sulphur and fermentable substances tending to interfere with the growth of some organisms.

4. For the filtration of broths and similar fluid media, heat a large-size

funnel in an Arnold sterilizer or with hot water. Place a small square of coarse wire netting in the funnel and cover the netting with a layer of good grade absorbent cotton. Moisten with a small amount of medium to "set" the cotton filter; then add the balance of the medium. Pour back the first filtrate several times until it comes through clear. Filter paper may be used with a fluted glass funnel (Fig. 165).

5. Agar and gelatin media must be filtered while hot and fluid. The funnel must be hot, as likewise the receiving flasks. The funnel may be kept hot by placing it in a hot water funnel (Fig. 166) to prevent congealing of the medium during filtration.

6. Filtration may be made directly into test tubes or flasks.

FIG. 165. — FLUTED GLASS FUNNEL FOR RAPID FILTRATION.

FIG. 166.—HOT WATER FUNNEL.

STERILIZATION OF CULTURE MEDIA WITH HEAT

1. Culture media may be sterilized by (*a*) heat (autoclave, Arnold sterilizer or water bath) or (*b*) filtration. Volatile disinfectants like chloroform may be used for the preservation of serum, ascites fluid, etc., but chemical disinfection has not as yet been perfected.

2. The minimum of heat should be used, as overheating may destroy growth-stimulating substances as well as caramelizing sugars.

3. *Autoclaves* should be equipped with thermometers, as the temperature is more reliable than pounds of pressure. In general terms the equivalents are as follows:

$$5 \text{ pounds pressure} \dots \dots \dots \dots 107.7° \text{ C. } (226° \text{ F.})$$
$$10 \text{ pounds pressure} \dots \dots \dots \dots 115.5° \text{ C. } (240° \text{ F.})$$
$$15 \text{ pounds pressure} \dots \dots \dots \dots 121.6° \text{ C. } (250° \text{ F.})$$
$$20 \text{ pounds pressure} \dots \dots \dots \dots 126.6° \text{ C. } (260° \text{ F.})$$
$$25 \text{ pounds pressure} \dots \dots \dots \dots 130.5° \text{ C. } (267° \text{ F.})$$
$$30 \text{ pounds pressure} \dots \dots \dots \dots 133.5° \text{ C. } (274° \text{ F.})$$

4. Two kinds of autoclaves are available: (*a*) the upright for small laboratories (Fig. 167) and (*b*) the horizontal (Fig. 168) for larger laboratories. The former is heated with gas, kerosene or electricity (special connection required); the latter may be heated with gas or connected with a steam plant. Those illustrated are recommended and full directions for operating are furnished by the manufacturers.

5. For media in test tubes, sterilization at approximately 121° C. for twenty minutes is sufficient; for media in bulk, thirty to sixty minutes are required.

6. With either autoclave allow time for agar to melt before timing the period of sterilization. Allow autoclave to cool before opening the door, as a sudden release or pressure may wet or blow the stoppers and crack glassware.

7. The *Arnold sterilizer* (Fig. 169) furnishes streaming steam at 100° C.
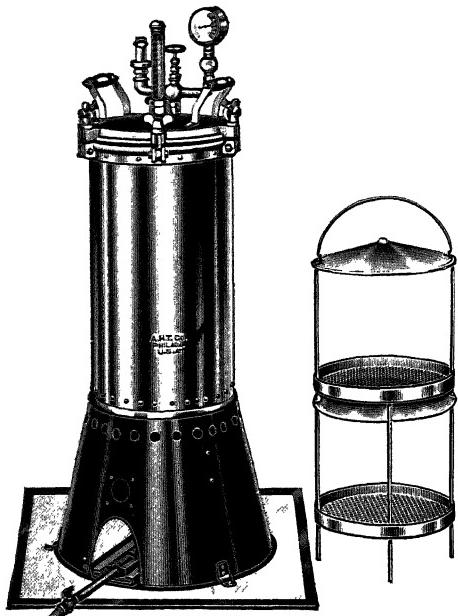
FIG. 167.—VERTICAL AUTOCLAVE.   FIG. 168 —HORIZONTAL AUTOCLAVE.
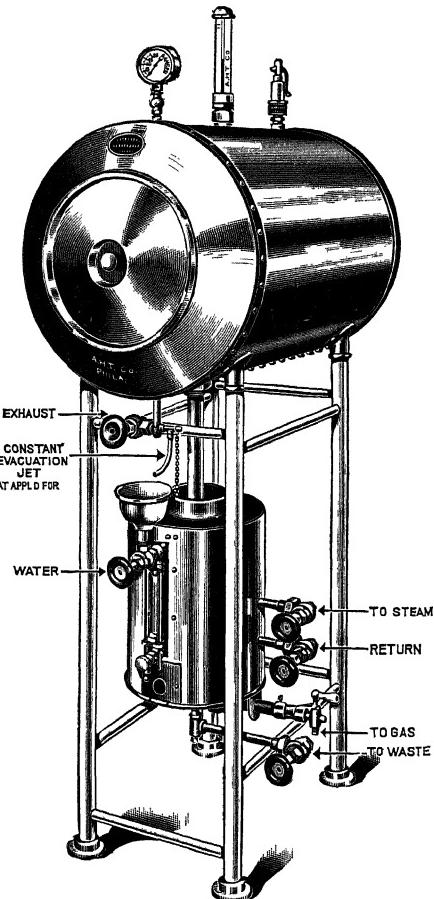
and is especially recommended for routine use, as overheating is readily avoided and likewise caramelization of sugars.

Make sure that there is plenty of water in the pan to avoid the possibility of boiling dry followed by the melting of soldered joints with danger of fire.

Media in test tubes should be heated for twenty minutes *after steam is produced*, and bulk media for forty-five to sixty minutes on each of three days in succession (*fractional sterilization*), allowing extra time for the melting of agar. Allow the sterilizer to cool before removing the contents.

8. *Water bath* sterilization is adapted only for small amounts of serum, ascites fluid, tissue extracts, etc., to prevent coagulation. The material should
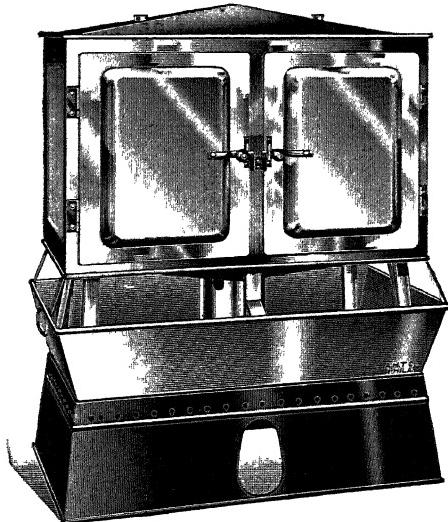
FIG. 169—ARNOLD STERILIZER OF DOUBLE DOOR TYPE.

be as free as possible from contamination. This method is valueless for killing spores. The water should be above the level of the fluid to be sterilized. The temperature should be 60° C. for about two hours.

STERILIZATION BY FILTRATION

1. Filtration methods are mainly employed for the removal of bacteria from serum, ascites and hydrocele fluids, tissue extracts, etc., that cannot be sterilized by heating at 60° C. (as when spores are present).
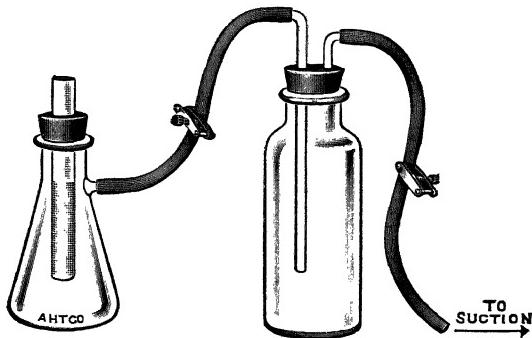
FIG 170—A SMALL FILTRATION APPARATUS.

FIG 171.—A WATER SUCTION PUMP

2. The filters are usually made of siliceous materials of negative electrical charge and with pores small enough to hold back bacteria and spores (Berkefeld

"W"=very fine; "N"=normal or medium, and "V"=coarse for rapid filtra-tion).

3. Suction or pressure must be provided. For small filters requiring the filtration of but small amounts of fluid, the apparatus shown in Figure 170 is usually sufficient, suction being provided by a suction pump attached to a faucet (Fig. 171).

4. For the filtration of small amounts permitting a control on pressure and
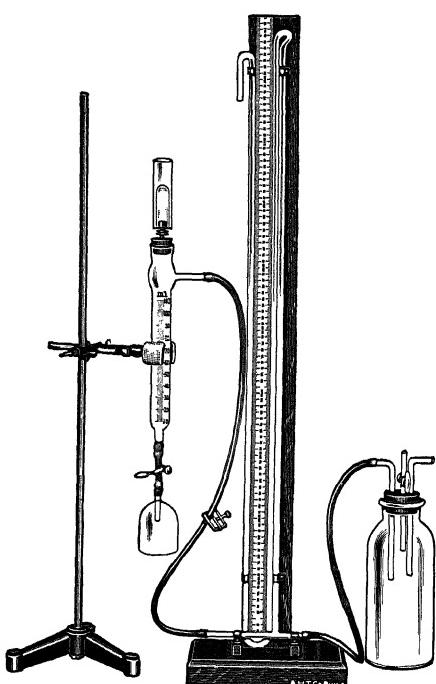
FIG. 172.—MUDD FILTRATION APPARATUS.
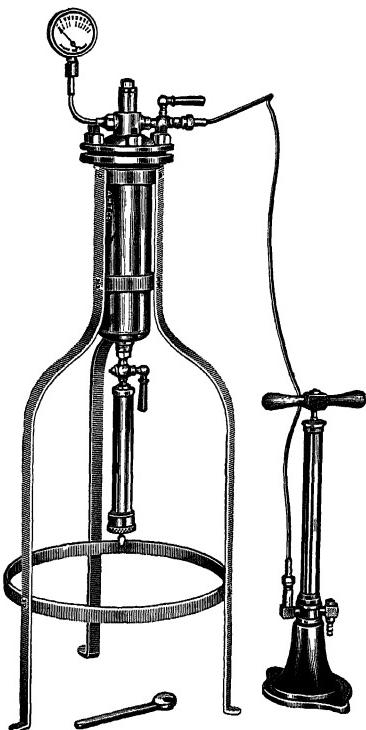
FIG. 173.—CHAMBERLAND-PASTEUR FILTER.

volume with the sampling of filtrate at any stage, the Mudd apparatus (Fig. 172) may be recommended.

5. For the filtration of large amounts, the Chamberland (Fig. 173), Mandler (Fig. 174) and Seitz (Fig. 175) filters may be recommended. The larger sizes of these require suction or pressure pumps. The Haen is a new form employing a membrane.

6. The filter candle and all attachments with which the filtrate will come in contact must be sterile. The glassware may be sterilized in a hot air oven; the candle and rubber connections may be boiled for an hour or autoclaved.

7. New candles should be cleansed before use by passing through distilled water, followed by placing in cold water and boiling for thirty to sixty minutes.

It is sometimes advisable to test for impermeability to bacteria with a broth culture of *B. prodigiosus.*

8. Paraffin, petrolatum, and other oils must be carefully avoided, as they tend to increase permeability to bacteria.

9. After use, candles should be cleansed by filtering through distilled water (to remove soluble and especially coagulable material) followed by sterilization by boiling (if infective material has been used) and a light scrubbing of the surface with a fine brush.

10. After continued use, candles become clogged (the average is approximately ten filtrations) and must be discarded. They may be heated to a glow, but this tends to produce cracks and increase their permeability.

11. Before candle filtration the material should be first filtered through a fine filter paper or paper pulp to remove large particles and reduce clogging.
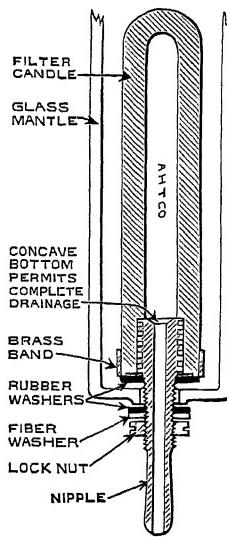
FIG. 174. — SECTIONAL VIEW OF MANDLER FILTER CYLINDER
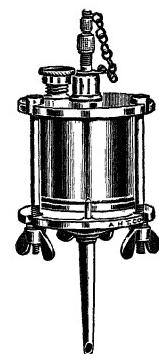
FIG. 175 — SEITZ-UHLENHUTH FILTER.

PREPARATION OF CULTURE MEDIA

General Directions.—1. Always use chemically pure (C.P.) reagents unless otherwise stated. Peptone and beef extract should be perfectly soluble. Sugars should be of highest purity.

2. Use distilled water unless tap water is indicated in the formula.

3. Weigh accurately.

4. Adjust reactions carefully so that the final P^H is optimum for the organism to be grown.

5. Sterilize strictly as directed.

6. Test all media for sterility by incubation at 37° C. for one or two days before storing or releasing for use.

7. Store culture media in a refrigerator to reduce evaporation to a minimum. After finishing such media as Löffler's blood serum and Dorset's egg, sterile distilled water may be added to cover the slants to prevent drying. The water is poured off before the medium is used. Agar slants may be freshened by placing the tubes in a water bath at 45° C. to bring moisture to the surface. Evaporation of tubed media after sterilization may likewise be prevented by removing the cotton stoppers and replacing them with boiled rubber stoppers.

8. Contamination (especially with molds) and drying may also be pre-

vented by paraffining the stoppers or covering them with rubber tissue, rubber caps or tin foil. The plugs of tubes may be dipped in hot paraffin. With flasks the excess cotton is trimmed off and melted paraffin (not too hot but almost ready to set) is poured on the top.

9. The large and complete list of various dehydrated culture media prepared by the Digestive Ferments Company of Detroit, Michigan, is satisfactory and can be highly recommended, especially for conservation of time as well as for reduced costs in production, particularly in the case of smaller laboratories. This firm also supplies sterile ascites fluid for enrichment purposes.

10. While a very large number of culture media have been described with numerous modifications in the technic of preparation, those given herewith are recommended for routine use. A few very special ones are described in later chapters in connection with the methods of cultivation of certain organisms.

11. For the identification of sugar media, Weidman has suggested adding colored beads, as red for glucose, blue for maltose, etc. Combinations may likewise be used and the cheap perforated beads are satisfactory.

BEEF EXTRACT BROTH

Beef extract (Bacto; Liebig's, etc.)	5 gm.
Peptone (Bacto; Witte, etc.)	10 gm.
Sodium chloride (optional)	5 gm.
Water	1000 c.c.

1. Mix; heat to dissolve the ingredients; boil ten minutes to throw down precipitates. Allow to cool.

2. Adjust the reaction to a little higher P^H than desired (usually 0.2 higher).

3. Add water to make up to original volume.

4. Filter through cotton and filter paper.

5. Sterilize at 121° C. (15 pounds pressure) for twenty to thirty minutes.

6. The P^H may be rechecked.

MEAT INFUSION BROTH

Chopped lean beef or veal	500 gm.
Water	1000 c.c.

1. Mix and allow to stand overnight in cold room or refrigerator.

2. Strain through cheesecloth and press the juice from the residue. The amount of fluid recovered should equal at least the amount of water added.

3. Boil until fluid is clear and meat particles are brown.

4. Strain through cheesecloth.

5. Filter through paper.

6. Add water to make 1000 c.c.

7. Titrate and adjust reaction.

8. Place in containers and sterilize at 121° C. (15 pounds pressure) for thirty minutes.

9. This medium may also be prepared as follows:

Bacto beef.............................. 100 gm.
Water (distilled)......................... 1000 c.c.

Heat at 50° C. for one hour.

Then heat at 80° C. for one hour to coagulate a portion of the proteins.

After straining through cheesecloth, add peptone and sodium chloride as above. Boil ten minutes; allow to cool and finish as in steps 2, 3, 4 and 5 above.

10. *Double strength broth* may be prepared by either of the above methods by using twice as much meat or Bacto beef and finishing as described. For use dilute with an equal part of sterile distilled water.

11. *Sugar-free beef infusion broth* may be prepared by removing muscle sugar by fermentation with *B. coli* as follows: (*a*) To 1000 c.c. prepared as above (steps 1 to 6) add 10 c.c. of a twenty-four-hour broth culture of *B. coli;* (*b*) incubate forty-eight hours; (*c*) heat in Arnold sterilizer for thirty minutes and then place in refrigerator; (*d*) fill two fermentation tubes and reinoculate with *B. coli*; (*e*) incubate one day; (*f*) if no gas is produced finish the medium as in steps 7 and 8 above.

HORMONE BROTH

Ground beef heart (lean)................... 500 gm.
Peptone (Bacto; Difco).................... 10 gm.
Salt....................................... 5 gm.
Gelatin.................................... 10 gm.
Tap water.................................. 1000 c.c.

1. Add 1 egg (shell included) slightly beaten.

2. Mix well and place in ice box overnight.

3. The following day heat the mixture over the flame slowly to 68° C., *constantly stirring,* and hold at that temperature for ten minutes when the meat will have turned brown.

4. Place in the Arnold sterilizer for one hour from boiling point or until clot is well formed.

5. With glass rod carefully loosen clot from sides of container and replace in Arnold for one to one and one-half hours, or until the coagulum settles, leaving a clear supernatant broth.

6. Place in the refrigerator overnight and next morning strain off meat and hardest fat by means of a fine wire sieve. Adjust the P^H to 8.5 to 9 by the addition of N/1 sodium hydroxide.

7. Siphon off this clear broth after waiting one-half to one hour until the

phosphates are precipitated. For stock medium store in liter containers, sterilizing for forty minutes on three successive days and adjusting the reaction later as put into use. For immediate use adjust P^H to 7.7 to 7.8 by means of normal hydrochloric acid; tube or flask as desired, and sterilize in Arnold for twenty to thirty minutes on three successive days.

8. The broth must not come in contact with any vegetable fiber or be heated above 100° C. at any time.

9. If it is desirable to remove the acid precipitate which will form in this medium when the reaction is lowered by the growth of bacteria, the following method can be used:

To stock medium before sterilization, add concentrated hydrochloric acid drop by drop, shaking between, till a maximum heavy white precipitate is formed. Place in Arnold sterilizer twenty minutes to throw down precipitate; siphon off the clear supernatant broth and store as stock or adjust reaction for use.

MEAT INFUSION AGAR

Agar (Bacto)	20 gm.
Peptone (Bacto; Witte, etc.)	10 gm.
Sodium chloride	5 gm.
Meat infusion broth	1000 c.c.

1. Shredded agar should be weighed, soaked in cold water for fifteen minutes and drained before adding to the medium. If powdered agar is used, it should be mixed with water to form a paste before adding.

2. Add the peptone and sodium chloride.

3. Heat at 121° C. for one hour.

4. Adjust the reaction.

5. Filter through cotton while hot. A hot water funnel may be used (see page 280). Repeat filtration if medium is not clear.

6. Fill into test tubes or flasks.

7. Heat at 121° C. for half hour.

8. If slants are desired, place the tubes while hot in a flat position. Raise the plugged end sufficiently to give the desired slant and allow to cool.

9. *Beef extract agar* may be prepared in exactly the same manner except that 20 grams of agar are added to each 1000 c.c. of beef extract broth. For the bacteriological examination of water and milk, the following special formula is recommended:

BEEF EXTRACT AGAR FOR BACTERIOLOGICAL EXAMINATION OF WATER AND MILK

(American Public Health Association, 1925)

Agar (Bacto)	15 gm.
Peptone (Bacto; Witte, etc.)	5 gm.
Beef extract (Bacto; Liebig's, etc.)	3 gm.
Water (distilled)	1000 c.c.

1. Dissolve the agar in 800 c.c. of water, heating in the autoclave for thirty minutes at 121° C.

2. Dissolve the peptone and beef extract in 200 c.c. of water.

3. Mix the two solutions.

4. Adjust the reaction to P^H 6.6.

5. Heat to 100° C. and filter through cheesecloth and cotton.

6. Fill into tubes or flasks and sterilize at 121° C. for twenty minutes.

GLUCOSE AGAR

Preparation is the same as for meat infusion agar (see page 288), except that 20 grams of glucose are added to each 1000 c.c. of the agar just before final heating. Sterilize by the fractional method.

GLYCEROL AGAR

Preparation is the same as for meat infusion agar except that 30 c.c. of C.P. glycerol are added before heating and adjusting the reaction.

BLOOD AGAR

Sterile blood............................... 10 c.c.,
Meat infusion agar......................... 90 c.c.

1. Melt the meat infusion agar (this should contain 2 per cent agar) by heat.

2. Allow the agar to cool to 45° C. Keep at this temperature until blood is added.

3. Have ready defibrinated or citrated horse, sheep, or human blood collected under sterile conditions. To defibrinate, collect in flask or bottle containing glass beads and shake until fibrin has formed. For citrated blood, place in the flask or bottle 1 c.c. of a sterile solution of 10 per cent sodium citrate for each 9 c.c. of blood collected. Human blood can be obtained from patients from whom blood is taken for the Wassermann test or blood chemistry. Withdraw 10 to 15 c.c. more than is required for the tests mentioned and immediately place in flask containing citrate solution.

4. Add 1 part of blood to 9 parts of agar. Have agar and blood heated to 45° C. at time of adding. Flame the mouth of flasks before pouring the blood, and mix thoroughly.

5. Pour into sterile Petri dishes, taking precautions to prevent contamination; allow to harden.

6. If slants are desired, the blood can be added to the agar in tubes or to agar in flasks and then pipetted into sterile tubes and slanted.

7. "Chocolate" blood agar is prepared in the same manner except that the blood is added to the agar at a temperature of 95° C., tubed and slanted.

ROSENOW'S GLUCOSE BRAIN BROTH

Dehydrated Bacto Nutrient Broth...	8 gm.
Sodium chloride.....	8 gm.
Dextrose (C.P.)	2 gm.
Andrade's indicator......................	10 c.c.
Water (distilled)...	1000 c.c.

Dissolve the broth and salt by heating. When cool, add the indicator and dextrose. Meat infusion broth may be used instead of the dehydrated broth. Tube in large tubes (20 by 1.5 centimeters), the column of broth to be about 12 centimeters deep. Add three pieces of calves' brain, about 1 centimeter square, also two or three pieces of calcium carbonate, preferably as crushed marble, to each tube. Sterilize for twenty minutes in the autoclave at 20 pounds pressure. If the broth is to be used for blood cultures, 5 grams of sodium citrate is added to each liter, to prevent coagulation of the blood.

ROSENOW'S GLUCOSE BRAIN AGAR

Dissolve 8 grams of powdered agar in 1 liter of glucose broth, prepared as above. Tube, add calf brain and calcium carbonate, and sterilize as in the case of the broth.

LIVER INFUSION AGAR

Ground beef liver........................	500 gm.
Agar (Bacto).............	20 gm.
Peptone (Bacto; Witte, etc.)...	10 gm.
Sodium chloride..........	5 gm.
Egg albumin.........	10 gm.
Water (distilled).	1000 c.c.

1. Mix the liver with 500 c.c. of water and heat at 100° C. for twenty minutes. Stir well and continue heating for ninety minutes.

2. Filter through wire sieve and then through glass wool.

3. Dissolve the peptone and salt in the infusion.

4. Dissolve the agar in 500 c.c. of water by heat and add to the infusion.

5. Add water to make 1000 c.c. If it is desired to inhibit gram-positive organisms, add 0.1 gram gentian violet (equals 1 : 10,000).

6. Adjust the reaction to P^H 7.0; or adjust to +0.2 per cent to phenol-phthalein for the gonococcus.

7. Dissolve the egg albumin in a little water and mix in thoroughly at a temperature of 50° C.

8. Heat at 100° C. for one and one-half hours.

9. Filter through wire sieve and glass wool.

10. Fill in tubes or flasks and heat at 121° C. (15 pounds pressure) for thirty minutes.

NOGUCHI'S RABBIT ASCITES AGAR

1. In long narrow test tubes (200 by 13 millimeters), sterilized, place small pieces of rabbit kidney removed under sterile conditions.

2. To 3 parts of melted agar (prepared of beef infusion broth with a P^H of 7.6) cooled to about 40° C., add 1 part of sterile ascites fluid.

3. Mix well and transfer to the sterile test tubes with a sterile pipet.

4. Incubate the tubes for at least forty-eight hours and discard any that may be contaminated.

5. As cooked kidney appears to give almost as good results as raw tissue, the tubes with kidney fragments may be sterilized at 121° C. (15 pounds pressure) for thirty minutes before the addition of the ascites agar.

6. *Rabbit serum agar* may be prepared in the same manner, using sterile human or horse serum instead of ascites fluid.

GONOCOCCUS MEDIUM OF PELOUZE AND VITERI

1. Pass 500 grams of calf brain through gauze into 500 c.c. of distilled water.

2. Place in refrigerator for twenty-four hours.

3. Filter through cotton several times (filtrate remains opaque).

4. Measure and to each 100 c.c. add 0.5 gram of acid sodium phosphate and 1 gram of peptone.

5. Add 1 part of this brain broth to 3 parts of 2.5 per cent veal broth agar with the addition of 0.5 per cent of sodium chloride and 1 per cent of peptone.

6. Adjust to P^H 7.8, which allows for a reduction of two points in auto-claving, as P^H 7.6 is desired.

7. Tube; heat at 121° C. (15 pounds pressure) for thirty minutes, and slant.

8. Replace the cotton stoppers with sterile rubber corks to retain the water of condensation.

ENDO'S MEDIUM (KENDALL'S MODIFICATION)

1. The base of this medium is beef extract agar with a P^H of 7.6. Melt the agar.

2. Add 5 c.c. of a sterile 20 per cent solution of lactose to each 100 c.c. of agar. (The lactose can be added to the agar before melting and in this way it is sterilized by the heating. Add 1 gram of lactose to each 100 c.c. of agar.)

3. Add 1 c.c. of sterile decolorized fuchsin to each 100 c.c. of agar. Decolorized fuchsin is prepared by adding 1 c.c. of a saturated alcoholic solution of basic fuchsin to 10 c.c. of a 10 per cent watery solution of sodium sulphite. Sterilize at 100° C. for twenty minutes.

4. Mix thoroughly and pour into sterile Petri dishes preferably with clay covers.

5. The medium should have a very pale pink or flesh color when hot. This disappears upon cooling. On standing several days the color gradually returns and for this reason it is necessary to prepare the medium fresh and use the same day.

SABOURAUD'S AGAR

Agar (Bacto)................................	15 gm.
Peptone (Bacto; Witte, etc.)...............	10 gm.
Maltose (C.P.).............................	40 gm.
Water (distilled)..........................	1000 c.c.

1. Mix and heat to dissolve.
2. Adjust reaction to +2.0.
3. Filter through paper.
4. Place in tubes or flasks and sterilize at 100° C. for thirty minutes on three successive days.

WEIDMAN'S MODIFICATION OF SABOURAUD'S AGAR

Agar......................................	18 gm.
Peptone (Fairchild's)......................	10 gm.
Dextrose (American granular)...............	40 gm.
Water.....................................	1000 c.c.

1. Dissolve ingredients and filter through cheesecloth.
2. Adjustment of P^H is unnecessary (the finished medium should have a P^H of 5.0).
3. Sterilize at 100° C. for thirty minutes on each of three days in succession.

RUSSEL'S DOUBLE SUGAR AGAR

Lactose (C.P.).............................	10 gm.
Glucose (C.P.).............................	1 gm.
Andrade indicator.........................	10 c.c.
Beef extract agar..........................	1000 c.c.

1. Melt the agar and add the other ingredients.
2. Mix and tube (fill more in the tubes than required for ordinary slants).
3. Heat to 100° C. for twenty minutes on three successive days.
4. Slant so there is a generous butt at the bottom (the slant should begin at least three-quarters of an inch from bottom of tube).
5. The Andrade indicator is prepared by dissolving 0.5 gram of acid fuchsin in 100 c.c. of distilled water. Add N/1 sodium hydroxide until the color changes to pink, then to brownish-red and finally to yellow (usually about 17 c.c.). Shake the reagent after each addition of the alkali.

LITMUS LACTOSE AGAR

Lactose (C.P.)............................ 10 gm.
Beef extract agar......................... 1000 c.c.
Litmus sufficient to give blue color

1. Dissolve the lactose in small amount of water and add to the melted agar.
2. Add sufficient litmus solution to obtain a blue color.
3. Fill in flasks and heat at 100° C. for twenty minutes for three successive days.

METHYLENE BLUE-EOSIN AGAR

Lactose (C.P.)............. . ... 5 gm.
Saccharose (C.P.)........... .... 5 gm.
Yellow eosin (sol. 2 per cent).. .... 20 c.c.
Methylene blue (0.5 per cent)............. 20 c.c.
Beef extract agar........................ 1000 c.c.

1. Add the lactose and saccharose to the agar and heat until agar is melted.
2. Add the eosin and then the methylene blue, mix and pour plates.
3. If this medium is to be used for isolating *B. dysenteriae,* the saccharose should be omitted.

SERUM GLUCOSE CYSTINE AGAR (FRANCIS)

Sterile serum........................... 25.0 c.c.
Glucose (C.P.).......................... 5.0 gm.
Cystine................................ 0.5 gm.
Meat infusion agar (PH7.4) .... 500.0 c.c.

1. Add the glucose and cystine to the agar and heat at 100° C. for fifteen minutes.
2. Cool to 50° C.
3. Add the sterile horse serum.
4. Tube and slant. Take precautions against contamination.

LÖFFLER'S BLOOD SERUM MEDIUM

Serum (horse, beef or sheep)................ 3 parts
Glucose broth (PH 6.8 to 7) .... 1 part

1. Mix the serum and broth (extract broth plus 1 per cent glucose) and tube.
2. Coagulate the medium, with the tubes in slanting position, by one of the following methods:

(a) Place the tubes in an inspissator (Fig. 176) and allow the temperature to rise slowly to between 80° and 90° C. Allow the tubes to remain at this temperature until coagulated.

(b) Or place the tubes in a slanted position in an Arnold sterilizer, leaving the door unlatched. Have the medium near the top of the sterilizer as the thermometer will give more nearly the temperature at which the medium is being heated. Allow temperature to rise to between 80° and 90° C., and leave tubes in until coagulated.

3. After coagulation the tubes can be sterilized by heating to 100° C. for twenty minutes on three successive days.

4. When coagulating do not heat over the temperature recommended and do not heat rapidly as the medium will be ruined with bubbles.

5. The serum used should be free from corpuscles and hemoglobin. Their presence does not spoil the medium although they render it brownish and unsightly in color.

6. Instead of the inspissator the following method may be employed: (a) Slant the tubes in an autoclave in baskets containing not more than three or four layers of tubes. (b) Close the door and close all outlet valves so that air cannot escape. (c) Turn on the steam and bring the pressure to 15 pounds for ten minutes. (d) Then open the outlet valve for the escape of air and steam but hold the pressure as constant as possible at 15 pounds. (e) When all air has escaped, close the valve and hold the pressure at 15 pounds for twenty minutes. (f) Close the steam valve and allow the pressure to drop to zero before opening the exhaust valves.
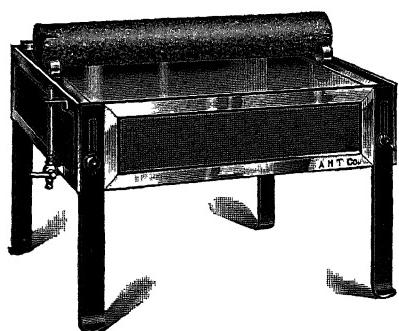
FIG. 176.—SERUM INSPISSATOR.

DORSET'S EGG MEDIUM

1. Clean four eggs by washing first with water and then with 5 per cent phenol solution. Allow to dry.

2. Open the eggs and place them in a sterile flask (holes can be punched into each end of the eggs with sterile forceps and the contents blown into the flask).

3. Add 25 c.c. of sterile distilled water.

4. Mix thoroughly and tube.

5. Proceed with coagulation and sterilization in the same manner as described for the preparation of Löffler's blood serum.

CORPER'S GLYCEROL CRYSTAL VIOLET POTATO MEDIUM

1. Large clean potatoes free from surface defects are cut into cylinders about 3 inches in length and five-eighths inch in diameter with a cork borer.

2. They are then halved longitudinally and soaked for one to two hours (until they acquire a faint blue tint) in 1 per cent sodium carbonate solution

(prepared from the pure anhydrous salt) containing crystal violet in concentration of 1 : 75,000 or 0.0015 per cent (the dye is added just before use as the mixture tends to decolorize).

3. They are then wiped with a clean towel and placed in sterile tubes (6 by ¾ inches) containing 1.5 c.c. of 6 per cent glycerol water.

4. Sterilize at 121° C. (15 pounds pressure) for thirty minutes (excessive heating to be avoided).

5. The above medium is used for the isolation of tubercle bacilli. For carrying pure cultures of the organisms, the following simpler medium may be prepared in the usual manner, tubed, autoclaved and slanted:

Mashed autoclaved potato....... .... 250 gm.
Glycerol.................... .... 25 gm.
Agar......................... . ... 15 gm.
Water..................... .... 710 c.c.

MILK (PLAIN)

1. Use fresh, unpasteurized milk.
2. Heat in Arnold sterilizer for thirty minutes.
3. Place in refrigerator overnight.
4. Remove the cream from the top or siphon off the milk from below the cream line.
5. Tube.
6. Heat at 100° C. for thirty minutes on three successive days.
7. *Litmus milk* is prepared in the same manner as plain milk except that a sufficient amount of litmus solution is added to produce a blue color just before tubing.

N. N. •N. MEDIUM

(*Nichols, Novy and MacNeal*)

Agar (Bacto)............................. 14 gm.
Sodium chloride......................... 6 gm.
Sterile rabbit blood..................... 300 c.c.
Water (distilled)........................ 900 c.c.

1. Add the agar and sodium chloride to the water and boil until agar is dissolved.

2. Tube and heat at 121° C. for thirty minutes. Keep for stock.

3. When needed, the number of tubes required can be melted and the sterile defibrinated rabbit blood added in the following proportion: agar, 2 parts, and rabbit blood, 1 part.

4. Mix thoroughly and slant.

POTATO MEDIUM

1. Select large white potatoes.
2. Scrub them thoroughly and pare each.

3. Wash in running water.

4. Cut into cylinders with a cork borer and then into wedge-shaped pieces. Each piece should have a good-sized base and be fairly large.

5. Wash in running water overnight or soak in 1 : 1000 solution of sodium carbonate for several hours.

6. Place each piece in a test tube with a small amount of cotton in the bottom.

7. Add water to cover butt but not the slant.

8. Heat at 121° C. (15 pounds pressure) for one-half hour.

9. *Glycerol potato medium* is prepared as in steps 1, 2, 3 and 4. Then soak the pieces overnight in 1 : 1000 sodium carbonate solution; drain and soak in 5 per cent glycerol for twenty-four hours; tube and add sufficient 5 per cent glycerol to cover the butts; sterilize as in step 8.

DUNHAM'S PEPTONE WATER

Peptone (Bacto; Witte, etc.) .... 10 gm.
Sodium chloride .... 5 gm.
Water....... .... 1000 c.c.

1. Mix and heat to dissolve peptone.

2. Fill in tubes or flasks and heat at 121° C. (15 pounds pressure) for one-half hour.

HISS'S SERUM WATER MEDIUM

Beef, sheep or human serum.............. 1 part
Water.................... .... 3 parts
Andrade indicator .... 1 per cent

1. Mix and add 1 per cent of any desired sugar. Heat at low temperature until sugar is dissolved.

2. Add Andrade indicator; mix, and tube.

3. Heat at 100° C. for twenty minutes on three successive days.

PETROFF'S MEDIUM

Ground beef............................ 500 gm.
Glycerin (C.P.)......................... 75 c.c.
Water................................. . 425 c.c.

1. Mix the glycerin and water; then add the meat. Allow to stand overnight in the refrigerator.

2. Squeeze the meat juice through gauze, towel or meat press and filter through cotton and paper and then sterilize by filtration.

3. Sterilize several eggs with alcohol and break them into a sterile flask or beaker. Beat them well with a sterile glass rod.

4. Mix one part of meat juice to two parts of egg.

5. To each 100 c.c. add 1 c.c. of a 1 per cent alcoholic solution of gentian violet.

6. Tube and coagulate by heating at 80° to 85° C. on three days for forty-five minutes.

7. Omit the glycerin if the medium is to be used for the isolation of bovine tubercle bacilli.

NORTH'S MEDIUM

Gelatin (Bacto).........	20 gm.
Peptone (Bacto; Witte, etc.)................	20 gm.
Double strength veal infusion...............	500 c.c.
Agar....................	10 gm.
Water..................................	500 c.c.

1. Dissolve the gelatin and peptone in the meat infusion.
2. Dissolve the agar in the water by heating and allow to cool to 50° C.
3. Mix the two.
4. Adjust the reaction to P^H 7.3 and heat at 100° C. for thirty minutes.
5. Filter.
6. Sterilize in the Arnold for forty-five minutes on three days in succession.
7. Prepare slants.

NUTRIENT GELATIN

Gelatin (Bacto).........................	100 gm.
Beef extract (Bacto; Liebig's, etc.)..........	3 gm.
Peptone (Bacto; Witte, etc.)..............	5 gm.
Water (distilled)........................	1000 c.c.

1. Mix and heat gradually until the gelatin is dissolved. Hold at 95° to 100° C. for fifteen minutes.

2. Adjust reaction to P^H 7.0.

3. Filter through hot paper pulp in a Buchner funnel or through filter paper after mixing with the white of an egg and heating to coagulate the albumin for clearing.

4. Tube and sterilize at 121° C. for fifteen minutes.

5. Place in refrigerator immediately after removing from sterilizer.

6. Excess heating of gelatin destroys its solidifying quality.

7. *Glucose gelatin* is prepared in the same manner except that 10 grams of glucose are added to each 1000 c.c. of medium.

CHAPTER XVIII

GENERAL BACTERIOLOGICAL METHODS

Principles.—1. While general bacteriology is an exceedingly complicated subject by reason of the very large number of bacteria known to exist, yet the number of bacteria producing disease of human beings and the lower animals is comparatively small and the majority are readily detected and identified by present-day methods.

2. Many may be identified by the proper staining of the exudates they produce, supplemented by a study of their cultures and biological characteristics. The technic is relatively simple but demands the employment of accurate methods, including proper methods of staining and differential staining.

3. A good microscope equipped with a satisfactory immersion or 1/12 objective and proper illumination are essential; it is a mistake to temporize with poor objectives, eyepieces and inadequate lighting.

PREPARATION OF SMEARS OF EXUDATES

1. The examination of stained smears of pus, sputum and other exudates is usually of value in bacteriological examinations and diagnosis; in some instances they constitute the chief means of diagnosis as in gonorrhea, Vincent's angina, spirofusillar gingivitis, acute contagious conjunctivitis, tuberculosis, leprosy, etc.

FIG 177—SMEAR WITH THIN AND THICK AREAS.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

2. Slides are preferred to cover glasses as they are less breakable, more easily handled and readily filed.

3. Smears may be prepared with cotton swabs or stiff wire loops. At least two should be prepared on the same or separate slides.

4. It is important to have smears neither too thick nor too thin. They need not be larger than a nickel if the material is scanty (Fig. 177).

5. *Vigorous rubbing should be avoided* as the cells may be broken up and intracellular examinations made difficult or impossible. This is especially important in examinations for gonococci and meningococci or when making a differential count of cells for cytodiagnosis. *The swab should be rolled on the slide* and should not cover the same area twice.

6. Cerebrospinal fluid and other transudates and exudates poor in cells may be first centrifuged and smears prepared of the sediment (Fig. 178).

7. In preparing smears of cultures, place a loopful of water on a slide; with a sterile wire transfer a small amount of culture to give an opalescent suspension; spread into a thin layer.
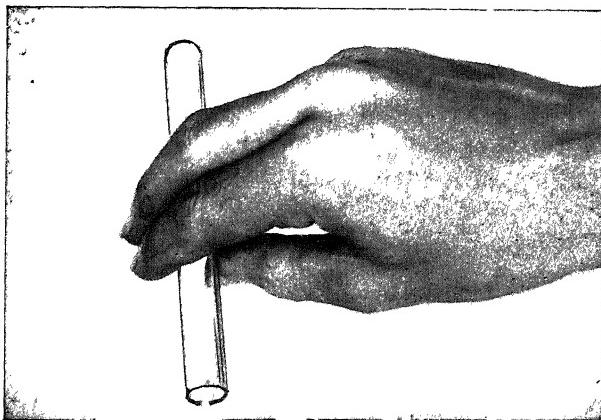
FIG 178—THOROUGHLY DRAINING SEDIMENT IN BOTTOM OF CENTRIFUGE TUBE BEFORE PREPARING SMEARS

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore)

8. Allow smears to dry in the air or with the aid of gentle heating. A slide may be dried by holding it *with the fingers* above a Bunsen flame, since
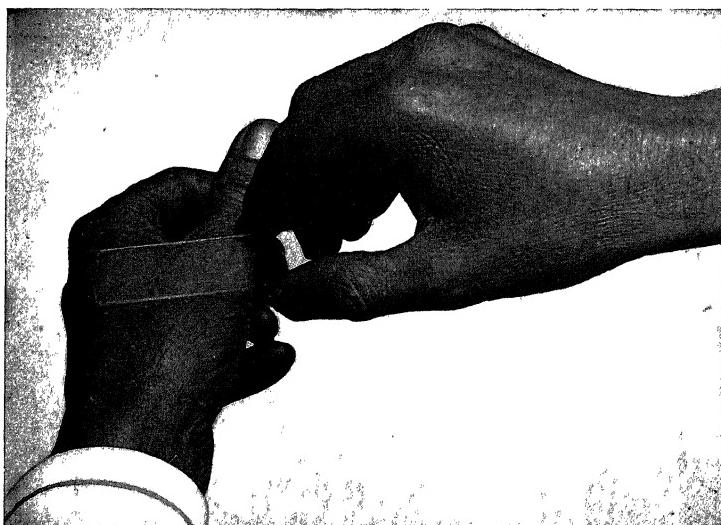
FIG. 179.—TOUCHING A HEATED SLIDE TO THE BACK OF THE HAND TO JUDGE THE TEMPERATURE AND AVOID "COOKING"

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore.)

a degree of heat bearable by the fingers will not "cook" or harm the smear (Fig. 179).

9. Do not use the filthy and dangerous method of covering a thick wet smear with another slide.

10. Dry unstained smears should be kept in a refrigerator until stained.

11. Stained smears keep indefinitely, but if mounted in Canada balsam, cedar oil, or dammar lac they tend to fade unless the preservative is *neutral*

PREPARATION OF CULTURES AND SUBCULTURES

1. A wire needle or loop is employed, held lightly as a pencil (Fig. 180). Sterilize immediately before and after making the transfer by first passing
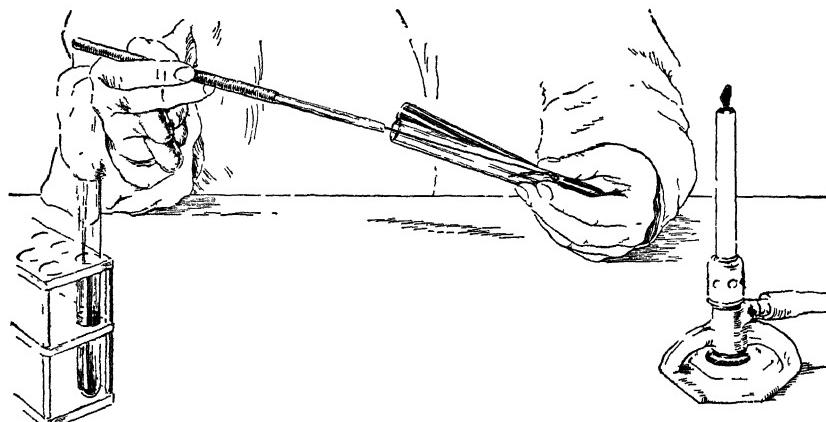
FIG 180—METHOD OF HOLDING TUBES AND WIRE FOR INOCULATION OF TUBES.

(From Wadsworth, *Standard Methods,* Waverly Press, Inc., Baltimore.)

the wire slowly through the flame of a Bunsen burner until it turns a dull red, then, more rapidly, the end of the handle to which the wire is attached (glass handles are very unsatisfactory because of cracking) ; the Kolle (Fig. 181) and Rosenberger and Greenman (Fig. 182) holders are recommended.

FIG 181.—KOLLE WIRE HOLDER.

FIG 182—ROSENBERGER AND GREENMAN WIRE HOLDER.

Platinum wire may be used but platinum-iridium is better and chromal wire (an alloy of nickel and chromium) is much cheaper and recommended because it is noncorrosive and resists destructive oxidation to a remarkable degree even at high temperatures.

2. The tubes should be held almost parallel with the table top to avoid air contamination (Fig. 180). Remove the plugs (do not flame) and hold them

between the third and fourth fingers of the right hand; now flame the ends of both tubes (but not too long, as cracking may occur) ; transfer the material; reflame the ends of the tubes and replace the stoppers. When making smears replace the plugs before spreading the material on the slide. It is not necessary to flame the stoppers before replacing them. If they are flamed, however, be sure to hold the test tube end of the plug low down in the flame to prevent the loose cotton held by the fingers from catching fire. Be sure that the plugs are inserted so deeply that they will not become loosened. Label properly and preferably with gummed labels as pencil markings may be rubbed off.
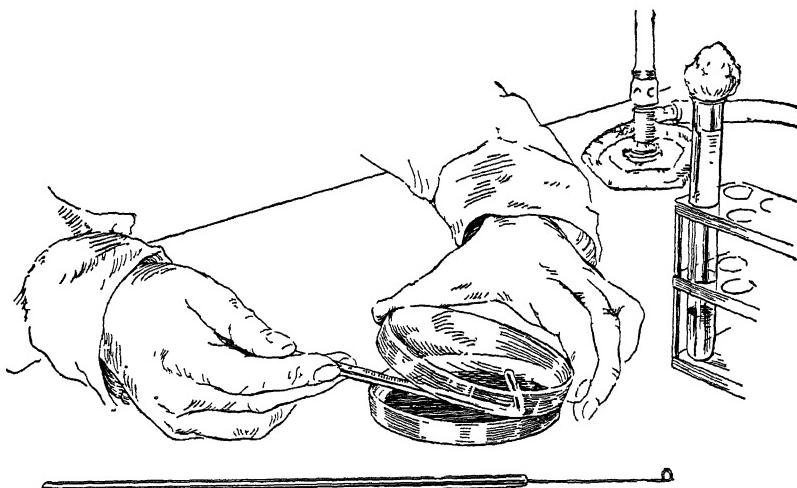
FIG 183 —METHOD USED IN SPREADING A DROP OF INOCULUM OVER THE SURFACE OF MEDIUM IN A PETRI PLATE BY MEANS OF A STERILE GLASS ROD

(From Wadsworth, *Standard Methods,* Waverly Press, Inc, Baltimore)

3. Be careful not to break the surface of a solid medium. The butt, however, may be punctured.

4. If gelatin is being used and if it is somewhat dry, it may be first melted in hot water and allowed to resolidify; inoculate with a deep puncture.

5. If the medium is semisolid, make a deep puncture with a loop, or if a pipet is used, expel the material slowly as the pipet is withdrawn.

6. If the medium is liquid, suspend the material in it with a loop or pipet.

7. When a large amount of fluid material is to be transferred, use a sterile pipet with a cotton plug. When the material is very infectious, attach a piece of rubber tubing with mouthpiece to the pipet or use a Pasteur pipet fitted with a rubber bulb. As soon as the culture has been made, place the pipet in a jar containing a disinfectant. If material is accidentally taken into the mouth, rinse thoroughly with water, then 40 to 50 per cent alcohol, 1 : 1000 bichloride of mercury, and again with water.

8. If a Petri plate is to be inoculated, raise the cover at one side just

high enough to admit the wire or swab, keeping the plate as completely covered as possible to prevent contamination from the air (Fig. 183).

Place a loopful of material on the medium and make successive streakings. After the first streak each succeeding one will deposit fewer organisms, so that the colonies developing from the last streaks should be well separated and suitable for isolating the various organisms present.

9. Another very good method is to deposit a small drop on the agar plate and spread it over the surface with a glass spreader, which can readily be made by bending a small glass rod at right angles at one end, using the longer end as a holder. If material contains many bacteria, it may be necessary to spread several additional plates with the same glass spreader without adding any more material.

ISOLATING AEROBIC BACTERIA

Shake or Pour Plates.— 1. Melt three agar tubes by boiling. (Be sure all is melted.)

2. Allow them to cool down to 42° C. and keep them at this temperature in a water bath until ready to inoculate.
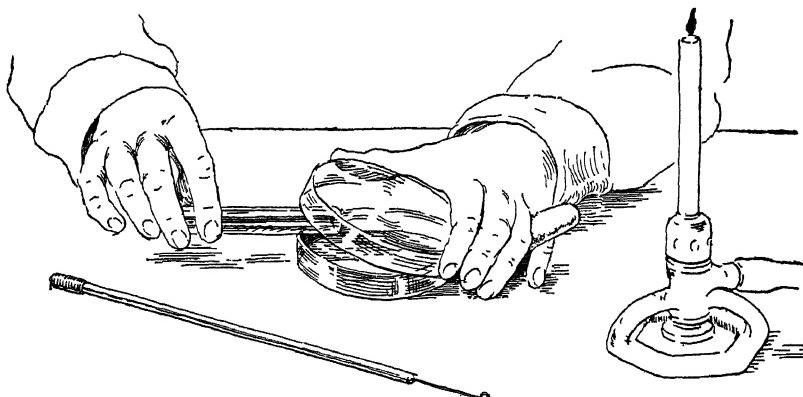
FIG. 184.—METHOD OF POURING INOCULATED MEDIUM INTO A STERILE PETRI PLATE.
(From Wadsworth, *Standard Methods,* Waverly Press, Inc., Baltimore.)

3. Into one of the tubes place a loopful of the material to be examined; shake well to insure uniform distribution. (Take care to prevent the formation of bubbles and contact of the medium with the plug.)

4. After flaming the wire, transfer three loopfuls from this tube to a second and mix thoroughly; repeat by placing five loopfuls into the third tube from the second. (This must be carried out rapidly as the agar may solidify before the transfers are completed and thus interfere with the next step.)

5. Pour the agar from each tube into a sterile Petri dish, taking care to flame the mouth of each and to lift the lids of the plates just enough to admit the end of the tube (Fig. 184); distribute evenly by gently rotating and tilting.

6. Allow the agar to solidify, and place the dishes in the incubator, cover side down.

7. If colonies have developed sufficiently at the end of twenty-four hours, examine both surface and deep colonies on whichever plates they are separated sufficiently to permit of fishing and subculturing.

8. Most of the colonies with this method of plating will be deep and very difficult to differentiate by their growth.

Surface Streak Plates.—1. If the material to be examined is not liquid, it should be emulsified in sterile salt solution or bouillon in a test tube, or if liquid and smears show organisms to be abundant it should be diluted.

2. Inoculate a wire loop with the material which has been emulsified or diluted and make eight or ten streaks over the surface of a blood agar plate or other medium which may be chosen. The loop should be allowed to rest lightly when being drawn over the agar to prevent cutting the surface.

3. After incubating a suitable length of time, examine the streaks for colonies.

The first streaks may be so thick with colonies as to prevent their study, but the last should have colonies well separated so as to permit of their study and isolation.

4. Another very good method is to place one to five loopfuls of the material on the surface of an agar plate and by means of a bent glass rod, which can be sterilized by flaming, spread the drop all over the agar. If many organisms are present, one or more plates may be smeared without placing any more material on the rod.

5. Or deposit a loopful on the center of a plate and streak with a wire loop in fanlike radiations, going back each time to the original area.

Isolation of Spore-Forming Organisms.—When material is known or thought to contain spores and is likely to be contaminated, a part, suitably diluted with sterile saline or broth, may be heated at 80° C. in a water bath for thirty minutes, or at 70° C. for one to two hours, to destroy the vegetative forms. Then proceed by any of the above methods for isolating pure cultures.

ISOLATING AND CULTIVATING BACTERIA ANAEROBICALLY

Anaerobic Jar Method.—1. First heat the medium (solid, semisolid or fluid) in boiling water for from fifteen to twenty minutes and cool to 40° to 42° C. before inoculating. This is to drive out oxygen.

2. Several anaerobic jars are available, like that of Novy (Fig. 185), Smillie (Fig. 186), and modifications of these. That similar to the McIntosh and Fildes jar is recommended.

3. The Novy jar depends upon the displacement of air with hydrogen alone while the Smillie and McIntosh-Fildes jars give displacement of air with hydrogen and the subsequent removal of any residual oxygen by oxidation of the hydrogen electrically heated by means of a small resistance coil sus-

pended just beneath the cover of the jar. Wadsworth has given the following directions for use:

(a) Place the plates (prepared as for aerobic growth) in the jar on a piece of wire gauze at the bottom to prevent breakage. Clean the opposing surfaces of the lid and the jar carefully with xylol and smear lightly with a grease composed of beeswax and vaselin. Grease also the tip and the threads of the needle valves of the gas inlet and outlet on the cover. Prepare an indicator by mixing in a test tube equal quantities of the following stock solutions: (1) N/160 sodium hydroxide; (2) 0.015 per cent aqueous methylene blue; (3) 6 per cent aqueous solution dextrose containing a few drops of chloroform or other preservative. Boil thoroughly until the methylene blue is reduced, as shown by the disappearance of color. Place the tube of indicator, while still colorless, in the jar, close immediately, and screw on the lid.
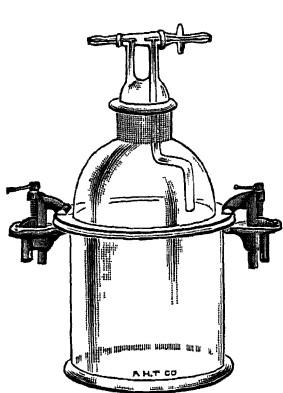
FIG 185—IMPROVED NOVY ANAEROBIC CULTURE APPARATUS.
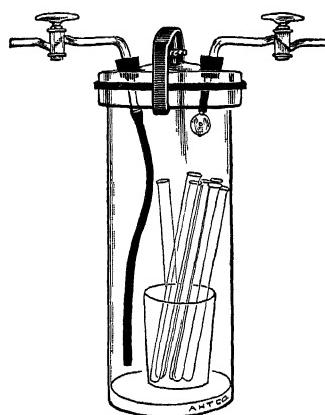
FIG 186—SMILLIE ANAEROBIC CULTURE APPARATUS

Avoid screwing down the lid too tightly as the pressure may break the jar. Attach the tube from the outlet of the hydrogen tank or generator to the gas inlet valve on the cover of the jar. (A tank of compressed hydrogen may be used as the source of supply, the flow being regulated by a reducing valve of the diaphragm type.)

(b) First, open the main valve on the hydrogen tank, then screw down the reducing valve until the pressure on the small gauge registers between one and two pounds. With both the inlet and outlet valves on the jar open, flood the jar with hydrogen at this pressure for one minute. Close the outlet valve on the cover of the jar, then immediately the inlet valve and then the reducing valve on the hydrogen tank. Disconnect the tube from the inlet valve on the jar. Connect the heating coil in the jar with the electric current through the required outside resistance, attaching the wires to the terminal posts on the cover of the

jar before plugging into the source of current. Leave the current on for ten minutes. (There is no flow of hydrogen during this period. In order to insure complete removal of any oxygen still present and to equalize the pressure, more hydrogen is now allowed to enter the jar.) Place one finger tightly over the tube from the hydrogen tank and open the reducing valve until the flow of hydrogen is just perceptible. The small gauge should barely register. Attach the tube to the gas inlet valve on the jar and open this valve, the outlet valve being kept closed. Continue the flow of hydrogen at this very slight pressure for from ten to fifteen minutes. During this period, allow the electric current to remain on as long as the top of the jar is cool. If the top becomes warm, turn the current off. Avoid excessive heating or prolonged heating of the coil in the jar as it shortens the life of the coil Close the gas inlet valve on the jar tightly and remove the tube connecting the hydrogen tank. Close the reducing valve and then the large valve on the hydrogen tank. Remove the plug connecting the outside resistance with the source of electric current and then the wires from the terminal posts on the cover of the jar.

(c) The methylene blue indicator remains colorless as long as perfect anaerobic conditions are maintained.

4. The *Brown anaerobic jar* is carefully and sturdily constructed and fitted with an all-metal lid, affords a maximum of safety, convenience and efficiency. It is essentially similar to the above and is used as follows:

The inoculated cultures (Petri dishes or tubes) are placed within the jar. A piece of "plasticine" (other modeling clays have been found less satisfactory) is rolled out in the form of a thin roll and placed onto the ground edge of the glass jar. The lid is pressed down onto the plasticine so as to make an air-tight joint and is held in place by the screw clamp. If a vacuum pump is available, a large part of the air may be pumped out of the jar. This serves to draw the lid down into place, to minimize the danger of explosion, and to establish anaerobic conditions more quickly. With the partial vacuum in the jar it is connected with the source of hydrogen which is led into the jar under a pressure of only 2 or 3 pounds. The terminals of the heating coil having been connected with an ordinary 110-volt electric light current which has been reduced by passage through a 50-watt light bulb, the current is turned on and allowed to flow until, by noting the collection of moisture on the sides of the jar, it is apparent that combustion of the hydrogen with the oxygen in the jar has taken place. It does no harm to allow the electric current to flow for twenty or thirty minutes. If the connections are tight and the hydrogen tank is provided with a reducing valve, the hydrogen may be allowed to flow into the jar throughout this period as fast as it is consumed, a pressure of 2 or 3 pounds being maintained by the reducing valve. When combustion is complete, the hydrogen inlet is closed, the electric current turned off and disconnected, and the jar is ready to be incubated. When used in this manner the atmosphere within the jar consists almost wholly of hydrogen. If it is desired to retain an atmosphere composed of nitrogen and a lesser amount of

hydrogen, the vacuum pump need not be used before the hydrogen is admitted and the electric current is turned on.

In using a vacuum pump it is an advantage to connect a graduated manometer by means of a T-tube with the rubber tube which leads from the pump to the jar. This permits reading the amount of vacuum obtained at any time. Also by this arrangement of apparatus it is possible to withdraw a measured amount of air from the jar and by means of the second gas cock in the lid of the jar to replace the evacuated air by an equivalent amount of carbon dioxide or other gas, a procedure useful in obtaining primary growths of certain bacteria, *e g.*, gonococcus and *B. abortus.* The fall or rise of the mercury in the manometer measures the amount of gas withdrawn or introduced.

When the jar is used for strictly anaerobic work, any residual oxygen which may have diffused out of the media within the jar after incubation for some time may be consumed by again passing the electric current through the coil, but it is unnecessary to introduce more hydrogen since there is always an excess within the jar.

Wright's Test Tube Method.—1. Boil glucose agar or gelatin to remove oxygen.

2. As soon as the media is sufficiently cool, inoculate with wire loop, making a deep stab nearly to the bottom of the tube.

3. Flame the cotton plug and push it down into the tube so that the top of the cotton is about three-fourths of an inch from the mouth of the tube.

4. Place a layer of pyrogallic acid about one-fourth inch thick on top of the cotton plug.

5. Add 2 or 3 c.c. of 10 per cent solution of caustic soda and insert a rubber stopper immediately.

6. Incubate.

7. To examine such cultures it may be necessary to loosen the entire culture from the sides of tube and remove it to a sterile dish where it can be cut or broken up to examine the growth. If gas is produced, cracks in the media may provide access to the growth and make it unnecessary to remove media from the tube.

Zinsser's Method.—1. Two crystallizing dishes 3 and 4 inches in diameter and 1 inch deep are used (one larger than the other). These should be sterilized.

2. Melt glucose agar by boiling and when cooled sufficiently (45° C.) inoculate material.

3. Pour the agar into the smaller dish and allow to harden. If surface growth is desired, uninoculated agar is poured in the plate and material spread on surface after it has hardened.

4. Place dry pyrogallic acid in the bottom of the larger dish.

5. Invert the smaller dish in the larger one.

6. Quickly pour a 5 per cent solution of caustic soda into space between

the sides of the two dishes (the smaller one inverted and the larger one upright) to a depth of one-half inch.

7. Immediately cover the soda solution with paraffin oil, which will act as a seal.

8. Incubate.

Wenzel Plate Method.—1. For the culture of blood, 2 c.c. of the citrated specimen are added to a tube containing about 15 c.c. of melted agar cooled to 40° C., and the mixture poured into a sterile Petri plate.

2. Cultures of swabs, pus and other material are prepared in the same manner except that several plates are used to secure isolated colonies.

3. The plates are chilled in an ice box until the agar is formed.

4. Sterile melted petrolatum (not too hot) is then poured over the surface of each to give a layer about 1 centimeter in depth.

5. Incubate in the usual manner.

6. For fishing, staining and transferring colonies, chill the plates and lift away the hardened petrolatum with a sterile wooden tongue depressor, scalpel or other suitable instrument.

Stitt's Method.—1. Make a deep agar stab in freshly sterilized glucose agar.

2. Place the culture and required amount of pyrogallic acid (1 gram per 100 c.c. of space) in a salt mouth bottle.

3. A rubber stopper perforated with a glass tube to which rubber tubing is attached is firmly pushed in.

4. Connect rubber tubing with vacuum pump and in five or ten minutes all air will be exhausted. Then clamp the rubber tubing with Hoffman clamp and screw it up tightly. Disconnect from pump.

5. Insert the tubing into a graduate filled with 10 per cent caustic soda. Unscrew the clamp and allow necessary amount of soda solution to be drawn in (1 gram per 10 c.c. of space). Screw the clamp up tight.

6. Incubate.

Increased Carbon Dioxide Tension Method.—To produce an atmosphere containing approximately 10 per cent of carbon dioxide, use a museum jar approximately 5 inches in diameter and 8 inches in height (inside measurements), which can be tightly sealed. Put the plates or tubes in the jar, then place 0.6 gram of sodium carbonate in the bottom of the jar. Pour over it a mixture of 1 c.c. of sulphuric acid and 10 c.c. of water (sufficient for a jar of approximately 2½ liters). When the reaction begins to subside, place an air-tight cover on the jar.

This method is particularly useful for the initial isolation of *B. abortus* (Bang).

Lowered Oxygen Tension Method.—Inoculate tall columns of brain-ascites agar or brain-hormone broth in 200 by 13 millimeter tubes by placing the material near the bottom, and incubate. The medium may be covered with

sterile vaselin or paraffin oil. The bacteria growing in the lower half of the tube are under lowered oxygen tension and sometimes give heavier growths than those near the surface.

METHODS FOR FISHING COLONIES

1. Remove the cover of the Petri plate and place it right side up on the table.

2. Examine the plate with un-aided eye or hand lens and ring off selected colonies with wax pencil on bottom of plate.

3. Or select colonies with the aid of the lower power of the microscope (Fig. 187).

4. With a sterile needle carefully remove portions of selected colonies to fresh media and prepare smears for staining. With adherent colonies and especially pneumococci and streptococci, it is sometimes neces-sary to cut out with a sterile loop a portion of the medium and transfer to broth.

Fig. 187.—Fishing and Transferring a Colony with the Aid of the Microscope.

(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

5. If the colonies are not well isolated, fish several to broth and prepare another set of plates.

6. In the case of tubes, flame the plugs thoroughly, heat the entire tube in a small flame just enough to soften the agar in contact with the glass, and then apply the flame to the bottom of the tube, expelling the cylinder of agar containing the colonies into a Petri plate. With a sterile knife or rod, cut the agar in sections so that the isolated colonies can be easily studied.

METHODS FOR STAINING BACTERIA

1. Stains should be kept in proper dropping bottles neatly labeled (Fig. 188).

Fig. 188.—Recom-mended Drop-ping Bottle.

2. Only sufficient stain for covering the smear should be used in the interests of economy. It may be spread with the tip of the bottle (Fig. 189).

3. For staining large numbers of slides various jars may be employed (Fig. 190) and the stain used many times (not advised, however, for staining tubercle bacilli). An excellent method is to place a rack of two glass or metal rods across a large slender dish for holding six or more slides to be stained at the same time.
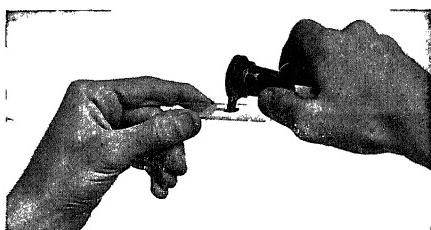
FIG. 189.—METHOD OF APPLYING STAIN TO A SLIDE.
(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore.)
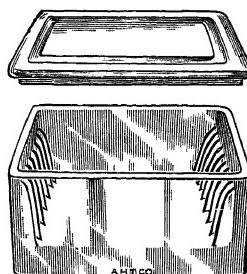
FIG. 190.—STAINING DISH.

Löffler's Methylene Blue.—1. Make thin smear of material to be examined on slide.

2. Dry in air and fix with gentle heat.

3. Cover smear with stain and allow to stand for two to five minutes; heat slightly if deep staining is desired.

FORMULA OF STAIN

Methylene blue (sat. alc. sol.)...... ...... 30 c.c.
Potassium hydroxide (0.01 per cent sol.).... 100 c.c.

4. Wash with tap water, blot and examine with oil-immersion lens.

5. Methylene blue does not stain very intensely and there is very little danger of overstaining. It is a very good stain to use when studying the morphology of organisms and is used especially in the examination of cultures for diphtheria bacilli.

Carbol Methylene Blue.—Same technic.

FORMULA OF STAIN

Methylene blue......................... 1.5 gm.
Alcohol (absolute).... .... 10.0 c.c.
Phenol (5 per cent) .... 90.0 c.c.

Carbolfuchsin (Ziehl-Neelsen).—1. Make thin smear on slide or cover glass.

2. Dry in air and fix by gentle heat.

3. Cover the smear with the stain, using it either diluted or undiluted de-

pending upon the intensity of staining desired. To dilute, cover the slide with water, then drop on the slide a drop or two of the stain or dilute 1 : 10 with water beforehand.

<center>FORMULA OF STAIN</center>

Method 1

Water (distilled)............................ 100 c.c.
Carbolic acid (crystals) .... 5 gm.
Alcohol (95 per cent)... .... 10 c.c.
Fuchsin.............. . ... 1 gm.

Method 2

Carbolic acid (5 per cent sol.).............. 100 c.c.
Fuchsin (sat. alc. sol.)......... .... 10 c.c.

4. Allow the stain to act for a minute or two. Heat may be applied if intense staining is desired.

5. Wash with tap water, dry, and examine with oil-immersion lens.

6. This is a very strong stain and overstaining may occur. The intensity of staining is best regulated by using proper dilution of stain, the degree of heat and time stain is allowed to act.

Gram's Method.—1. Make thin smear of material on glass slide or cover glass.

2. Dry in air and fix with gentle heat.

3. Cover smear with gentian violet solution (Weigert's) for two minutes.

<center>WEIGERT'S GENTIAN VIOLET</center>

Solution No. 1

Gentian violet............................ 2 gm.
Aniline oil................................ 9 c.c.
Alcohol (95 per cent)..................... 33 c.c.

Solution No. 2

Gentian violet............................ 2 gm.
Water (distilled).... .... 100 c.c.

Mix 1 c.c. of solution No. 1 with 9 c.c. of No. 2 and filter. The stock solutions Nos. 1 and 2 keep indefinitely, but when mixed to make staining solution it does not keep well and becomes unsatisfactory in about two weeks. It is well to mix and filter only enough of Nos. 1 and 2 to last two weeks.

4. Wash with water.

5. Cover with Gram's iodine solution, given p. 312, for one minute.

GRAM'S IODINE SOLUTION

Iodine. . . . . 1 gm.
Potassium iodide. . . . . 2 gm.
Water (distilled). . . . . 300 c.c.

Keep in dark bottle.

6. Pour off excess and wash with acetone (Fig. 191) until smear ceases to lose color (few seconds).

FIG. 191.—METHOD OF DECOLORIZING WITH ACETONE.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc, Baltimore.)

7. Wash with water.
8. Counterstain with 1 : 10 solution of carbolfuchsin for one minute.
9. Wash with water.
10. Dry and examine with oil-immersion lens.
11. The gram-positive organisms will appear dark-violet or purple; the gram-negative organisms will be colored a faint pink by the fuchsin. Occasionally capsules may be demonstrated with this method.
12. Counterstaining may also be conducted with a 2 per cent solution of safranine O in water or a 1 per cent solution of Bismarck brown in water.

Burke's Method of Gram Staining.—1. Air dry thinly spread film and

fix with least amount of heat necessary to kill the organisms and fix them to the slide. The films may be prepared with either tap or distilled water or saline solution.

2. Flood the smear with a 1 per cent aqueous solution of crystal violet. Mix with the dye on the slide 3 to 8 drops of a 5 per cent solution of sodium bicarbonate and allow to stand two to three minutes.

3. Flush off the excess stain with Gram's iodine solution (iodine, 1 gram; potassium iodide, 2 grams, and distilled water, 100 c.c.) and cover with fresh iodine solution; let stand for one minute or longer.

4. Wash in water and blot off all free water until the surface of the film is *practically free of water, but do not allow the film to become dry.*

5. Decolorize with pure acetone until the acetone flows from the slide practically uncolored (requires less than ten seconds).

6. Blot dry. The slide quickly dries without blotting.

7. Counterstain for five to ten seconds or longer if desired with a 2 per cent aqueous solution of safranine O.

8. Wash off excess stain by short exposure to water, blot and dry.

9. The slide may be immersed in xylol or turpentine for several minutes or until clear. Examine.

10. If the stain is unsatisfactory, wash off the oil with xylol, wash off the xylol with acetone, and restain.

Rapid Method for Staining Acid-Fast Bacilli.—1. Prepare thin (but not too thin) smears of material on slide or cover glass.

2. Dry in air and fix by gentle heat.

3. Cover smear with undiluted carbolfuchsin, gently heating to steaming for five minutes (do not boil); allow to cool until a precipitate forms (requires about two minutes; essential).

4. Wash in tap water.

5. Decolorize with acid alcohol; 15 per cent sulphuric acid or 30 per cent nitric acid in water may be used instead of acid alcohol.

ACID ALCOHOL

Hydrochloric acid (C.P.) .... 3 c.c.
Alcohol (95 per cent).. .... 97 c.c.

6. Wash in water.

7. Counterstain with Löffler's methylene blue for two minutes.

8. Wash in water.

9. Dry and examine with oil-immersion lens.

10. The acid-fast organisms will appear red; all other organisms and cells will appear blue. This method of staining is extensively used for detecting the tubercle bacillus in sputum and other material. Spores will also retain the red stain, and this method may be used to demonstrate their presence.

11. Counterstaining may also be conducted with a saturated alcoholic solution of picric acid (acid-fast bacilli pink in a soft yellow background).

12. The average number of acid-fast bacilli per field may be recorded according to the following scheme of Gaffky as modified by L. Brown:

No. 1. Only one to four in a whole preparation
No. 2. Only one bacillus on an average in many fields
No. 3. Only one bacillus on an average in each field
No. 4. Two to three bacilli on an average in each field
No. 5. Four to six bacilli on an average in each field
No. 6. Seven to twelve bacilli on an average in each field
No. 7. Thirteen to twenty-five bacilli on an average in each field
No. 8. About fifty bacilli on an average in each field
No. 9. One hundred or more bacilli on an average in each field
No. 10. Enormous numbers in each field

Cold or Slow Method for Staining Acid-Fast Bacilli.—1. Prepare, dry, and fix smears as in rapid method.

2. Place them in a staining dish filled with carbolfuchsin in an incubator for fifteen to twenty-four hours. (There is some danger of tubercle bacilli in sediment adhering to negative slides.)

3. Place in refrigerator for one-half hour for precipitation.

4. Wash in water, decolorize and counterstain as in rapid method.

Safranine Method (Ransom).—1. Prepare, dry, and fix smears as in the rapid method.

2. Cover with safranine stain (100 c.c. distilled water; 1 c.c. of normal sodium hydroxide and 4 grams of safranine). Heat to steaming for several minutes.

3. Wash in water.

4. Decolorize, counterstain, wash and mount as in the rapid method.

Pappenheim's Method of Differentiating the Tubercle and Smegma Bacillus.—1. Fix smears with heat.

2. Stain with hot carbolfuchsin for two minutes.

3. Pour off stain; do not wash; pour on and off four or five times the following mixture: rosalic acid, 1 gram; absolute alcohol, 100 c.c.; methylene blue to saturation; when these are mixed, add 20 c.c. of glycerol.

4. Wash in water and dry. The smegma bacilli are decolorized; tubercle bacilli are red, but the method is of questionable value and is not highly recommended.

Neisser's Stain for Diphtheria Bacilli.—1. Prepare smear in usual manner, fix with heat and place in solution No. 1 for two or three seconds.

SOLUTION No. 1

Methylene blue (Grubler) .... 0.1 gm.
Alcohol (96 per cent)..................... 2.0 c.c.
Glacial acetic acid....................... 5.0 c.c.
Water (distilled)......................... 95.0 c.c.

Dissolve the methylene blue in alcohol and add to the acetic acid and water.

2. Wash in tap water.

3. Place in solution No. 2 for three to five seconds.

SOLUTION No. 2

Bismarck brown........................... 0.2 gm.
Water (boiling)........   .... 100.0 c.c.

Dissolve the stain in boiling water and filter through filter paper.

4. Wash, dry, and mount.

5. The bacilli may stain uniformly brown or may show at one or both ends a dark blue, round body. True diphtheria bacilli usually show the blue bodies, while the pseudotypes show few if any.

Giemsa's Method.—1. Fix smears in neutral methyl alcohol for one minute.

2. Cover slide with Giemsa stain diluted as follows: Add one drop of a 1 per cent solution of potassium carbonate to 10 c.c. of distilled water and then add 10 drops of the stain (one drop of stain to each c.c. of alkaline distilled water).

3. Allow the stain to act for one-half to three hours or overnight (the longer the better).

4. Pour off stain and wash in running tap water for two or three minutes.

5. Dry with filter paper and examine:

Nuclear substance: blue, red or azure
Bacteria: dark purple
Metachromatic granules: azure
Cytoplasm of protozoon: blue

The stain can be purchased ready for use from any laboratory supply house, or be prepared as follows:

Azure II-eosin.................. .... 3.0 gm.
Azure II...................... .... .. 0.8 gm.
Glycerin (Merck's) (C.P.).......... . .... 250.0 c.c.
Methyl alcohol (C.P.)................. . 250.0 c.c.

Heat alcohol and glycerin to 60° C. Put the dyes in the alcohol and slowly add the glycerin, stirring. Stand at room temperature overnight and filter.

METHODS FOR STAINING SPIROCHAETA PALLIDA (TREPONEMA PALLIDUM) AND OTHER SPIROCHETES

Fontana's Method.—1. Make thin smear and dry without heating.

2. Cover smear with Ruge's solution for one minute for fixation.

RUGE'S SOLUTION

Glacial acetic acid.......... .... 1 c.c.
Formalin (40 per cent).................... 20 c.c.
Water (distilled)........................ 100 c.c.

3. Wash with water and blot.

4. Cover slide with alcohol and ignite.

5. Cover smear with mordant and warm gently until steam arises and then allow to act for thirty seconds.

MORDANT

Tannic acid...........	5 gm.
Phenol...................................	1 c.c.
Water (distilled)	100 c.c.

6. Wash with water and blot.

7. Cover smear with Fontana's solution and gently warm until steam arises and allow to act for thirty seconds.

FONTANA'S SOLUTION

To a 5 per cent silver nitrate solution, add ammonia drop by drop with a capillary pipet until a dark precipitate forms and redissolves. Then add more silver nitrate solution until the solution is slightly cloudy.

8. Wash, dry, and mount.

9. The spirochetes are stained dark brown or black.

Silver Impregnation Method.—1. Dry the smears in an incubator for several hours.

2. Place them in 10 per cent water solution of silver nitrate for several hours (Flexner advises three or four days) in diffuse sunlight until a brownish color with a metallic sheen develops.

3. Wash well in water, dry, and mount.

4. The spirochetes are a deep black color on a pale brown or colorless background.

Burri's India Ink Method.—1. Take one loopful of secretion from a chancre or other material to be examined and place on one end of glass slide.

2. A small quantity of India ink (flüssige Perltusche) is mixed with the secretion on the slide. This can well be done by dipping a match stick or toothpick in the ink and transferring a small amount to the slide and mixing it with the secretion.

3. After mixing, make a smear in same manner as described for blood.

4. Allow to dry and examine with oil-immersion lens.

5. The spirochetes will appear as white spirals against a dark background.

METHODS FOR STAINING SPORES

Acid-Fast Stain.—The method described for staining acid-fast bacteria (see page 312) may be employed for staining spores. By this method spores are stained red and bacillary bodies blue.

Huntoon's Method.—1. Make a rather thick smear.

2. Dry and fix with heat.

3. Cover with stain and steam for one minute.

STAIN

Solution No. 1

Acid fuchsin (Grubler)....................... 4 gm.
Acetic acid (2 per cent sol.).................. 50 c.c.

Solution No. 2

Methylene blue (Grubler).................... 2 gm.
Acetic acid (2 per cent).................... 50 c.c.

Mix equal parts of the two solutions; set aside for fifteen minutes; filter out heavy precipitate. Use filtrate for stain (keep several weeks); refilter if necessary.

4. Wash in water (film appears bright red).

5. Dip slide in dilute solution of sodium carbonate (7 or 8 drops saturated solution in tumblerful of water). When film turns blue, rinse immediately in water.

6. Dry and examine (spores red; body of bacillus blue).

METHODS FOR STAINING CAPSULES

Gram's Stain.—For Gram's method, see page 310.

Welch's Method.—1. Cover film with glacial acetic acid for few seconds.

2. Drain and replace with aniline gentian violet. Drain and again replace with aniline gentian violet. Repeat until all acid has been replaced by gentian violet solution.

3. Wash in a 1 or 2 per cent solution of sodium chloride and mount in same. Do not use water at any stage.

4. The capsule stains pale violet.

Hiss's Copper Sulphate Method.—1. Grow organisms in ascitic fluid or serum medium or mix with drop of serum and prepare smears.

2. Air dry and fix with heat.

3. Cover preparation with 5 or 10 per cent water solution of gentian violet and heat for few seconds until steam arises (5 c.c. of saturated alcoholic solution of gentian violet to 95 c.c. of·distilled water).

4. Wash dye off with 20 per cent solution of copper sulphate crystals.

5. Blot and dry thoroughly.

Huntoon's Method.—1. Mix the organisms with a small drop of nutrose solution on a slide and spread the film; dry in air (do not fix).

NUTROSE SOLUTION

Nutrose (sodium caseinate)........ ...... 3 gm.
Water .... 100 c.c.

Cook the solution for one hour in Arnold sterilizer and add 0.5 per cent carbolic acid. Place in tubes without filtering.

2. Cover with stain for thirty seconds.

STAIN

Carbolic acid (2 per cent aq. sol.)........... 100.0 c.c.
Acetic acid (1 per cent aq. sol.)............. 1.0 c.c.
Lactic acid (conc.)....................... 0.5 c.c.
Carbolfuchsin............................ 1.0 c.c.
Basic fuchsin (sat. alc. sol.)............... 1.0 c.c.

3. Wash in water and dry.

METHODS FOR STAINING FLAGELLA

Preparation of Film.—1. Place enough of an eighteen-hour culture in a few c.c. of filtered tap water to produce slight cloudiness. This may be used immediately or placed in an incubator for one or two hours to permit slight development.

2. Place a very small drop of the suspension on *a most carefully cleaned slide*; spread and allow to *dry quickly*. The less the suspension is manipulated the better. If drying is slow the bacteria may shed their flagella; if handled roughly they will be broken off.

Pittfield Method (Muir's Modification).—1. Cover film with mordant and steam gently for one minute.

MORDANT

Tannic acid (10 per cent aq. sol.)............ 10 c.c.
Mercuric chloride (sat. aq. sol.)............. 5 c.c.
Alum (sat. aq. sol.)....................... 5 c.c.
Carbolfuchsin....... ... ... 5 c.c.

Allow the precipitate to settle or it may be centrifuged. This solution will keep only about one week.

2. Wash well in water for about two minutes.
3. Dry in the incubator (do not blot with paper or cloth).
4. Apply stain, and heat gently for one minute.

STAIN

Alum (sat. aq. sol.)....................... 10 c.c.
Gentian violet or carbolfuchsin (sat. alc. sol.).. 2 c.c.

This stain does not keep over two days.

5. Wash with water, dry, and mount.

Löffler's Method (Kulp's Modification).—1. The bacillus is cultivated for at least two generations on agar slants having condensation water. Transfers are made from the condensation water.

2. Transfer 1 or 2 drops of condensation water of final culture to a tube of 15 to 20 c.c. of sterile water and incubate forty-eight hours.

3. Place a large drop at one end of a very clean slide. Hold vertically and allow the drop to run down the slide. Place in incubator for quick drying.

4. Apply mordant for fifteen minutes at room temperature.

<div align="center">MORDANT</div>

<div align="center">

Tannic acid (20 per cent aq. sol.)............. 50 c.c.
Ferrous sulphate (sat. aq. sol.)............... 25 c.c.
Basic fuchsin (sat. 95 per cent alc. sol.)....... 5 c.c.

</div>

5. Wash carefully.
6. Stain for fifteen minutes at room temperature.

<div align="center">STAIN</div>

<div align="center">

Aniline oil (sat. aq. sol. freshly filtered)....... 100 c.c.
Basic fuchsin (sat. 95 per cent alc. sol.) ...... 12 c.c.

</div>

7. Wash off excess stain and allow slide to dry.

METHODS FOR STAINING ORGANISMS IN TISSUES

1. Obtain tissue as soon as possible after death to prevent postmortem changes.

2. Place blocks not larger than one-quarter by one-eighth inch in Zenker's fluid for three to twelve hours.

3. Wash in water for several hours.

4. Place for twenty-four hours in each of the following alcohols in succession: 30, 60, 90 per cent and absolute.

5. Place in cedar oil or xylol until translucent.

6. Place in equal parts of cedar oil or xylol and paraffin at 37° C. for two hours.

7. Place in paraffin at 52° C. for two hours in each of two baths.

8. Box and cut sections of 3 to 6 micra.

9. Dry sections in incubator for about twenty-four hours or overnight.

10. Remove the paraffin by placing the slides in xylol and then in absolute alcohol. Repeat until all paraffin is removed and then place sections in water (no clouding denotes removal of paraffin).

11. Stain in Löffler's methylene blue for five to thirty minutes.

12. Decolorize for a few seconds in 1 per cent acetic acid.

13. Place in absolute alcohol and then in xylol and mount in Canada balsam.

Goodpasture's Stain.—This method is highly recommended.

1. After step 10 place the sections in the stain for ten to thirty minutes:

Alcohol (30 per cent) .... 100.00 c.c.
Basic fuchsin..................... .... 0.59 gm.
Aniline oil................... . ... 1.00 c.c.
Phenol (crystals)..................... 1.00 gm.

2. Wash in water.

3. Place in 40 per cent formalin for a few seconds (bright red color fades to a clear rose).

4. Wash in water.

5. Counterstain in saturated water solution of picric acid for three to five minutes (until section assumes a purplish-yellow color). Wash in water.

6. Differentiate in 95 per cent alcohol (red appears and some is washed out; some picric acid is washed out).

7. Wash in water.

8. Stain in Sterling's gentian violet for five or more minutes:

Gentian violet........................... 5 gm.
Alcohol (95 per cent).................... 10 c.c.

Grind in a mortar and add:

Aniline oil................... .... 2 c.c.
Water (distilled)................... ...... 88 c.c.

Stand for one or two days and filter.

9. Wash in water.

10. Place in Gram's iodine solution for five minutes.

11. Blot dry without washing.

12. Place in aniline oil and xylol (equal parts) until no more color comes away.

13. Place in two changes of xylol.

14. Mount in Canada balsam.

15. Gram-negative organisms stain red; gram-positive organisms, blue; tissues stain in shades of red to purple.

METHODS FOR STUDYING UNSTAINED BACTERIA

Hanging Drop.—1. Smear a little vaselin or immersion oil around the edge of the hollow space on a hanging drop slide (Fig. 192).

2. Place a loopful of fluid containing the bacteria in the center of a cover glass and immediately invert it over the hollow space on the slide, resting it on the vaselin.

FIG. 192.—HANGING DROP SLIDE.

3. Press the cover slip gently to spread the vaselin and to make a perfect seal. The purpose of the vaselin is to keep the cover slip from sliding and the fluid from evaporating.

4. Examine with either 1/6 or oil-immersion objectives. If examining only for motility, the lower power usually suffices. Care should be taken

FIG 193—OUTFIT FOR DARK-FIELD EXAMINATION FOR SPIROCHETES
(From Kolmer, *Chemotherapy and the Treatment of Syphilis*, W. B Saunders Co)

when focusing not to break the cover glass. The light should be considerably reduced with the diaphragm.

Dark-Field Illumination.—1. Apparatus necessary (Fig. 193): strong illuminating lamp such as a small arc lamp (Fig 10) or high-power incandescent lamp; funnel stop to be placed in oil-immersion objective to cut out rays which interfere, and a special substage condenser (Fig. 194; also see Fig. 6).
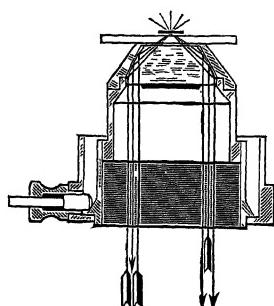
FIG 194—SECTIONAL VIEW OF SPECIAL DARK-FIELD CONDENSER

2. Remove substage condenser from microscope and adjust dark-field condenser in its place.

3. Insert funnel stop in the oil-immersion objective.

4 Center the apparatus with low-power lens by getting concentric rings on the upper surface of the dark-field condenser and adjusting by means of the three centering screws on edge of the condenser until the rings are parallel with the circle of the microscopic field.

5 Place a small drop of material to be examined on a scrupulously clean

slide (1 45 to 1 55 millimeters thick) and cover with clean cover glass (avoid air bubbles).

6. Place large drop of oil on top of condenser and put slide on stage so the oil forms a contact with the under surface of the slide.

7. Place oil on top of cover slip and examine with oil-immersion objective Examination can also be made with low and high power objectives.

METHODS FOR STUDYING BIOLOGICAL PROPERTIES OF BACTERIA

Study of Colonies.—1. The appearance of colonies is of aid in identifying bacteria.

2. As a rule, young colonies are most characteristic.

3. Colonies growing in the depths of plate may appear differently from those on the surface.

4. By naked eye inspection or with the aid of a hand lens, colonies may be:

(*a*) Moist or dry; adherent to medium; mucoid or slimy
(*b*) Transparent or opaque
(*c*) Edge sharply defined or indefinite
(*d*) Outline regular or irregular; fringelike
(*e*) Color white, yellow, pink, etc.
(*f*) Flat, raised or umbilicated

5. By microscopic examination (low power) finer details may be studied, like presence or absence of granules; granules fine or coarse and their arrangement, etc.

6. Appearance of colonies in fluid media:

(*a*) If pellicle: tenacious or easily broken up; thick or thin: stalactite formation
(*b*) Clear or cloudy and degree of clouding
(*c*) Sediment or deposit on sides of tubes; granular, flaky, gelatinous, mucoid

Pigment Production.—1. Many bacteria are capable of producing pigments of various colors which are of interest on account of their value in identifying these organisms. The pigmenting organisms of particular value on account of their pathogenic properties are:

Staphylococcus citreus· yellow pigment
Staphylococcus aureus: orange pigment
B. pyocyaneus: blue and green pigment
B. prodigiosus: red pigment

2. Under certain conditions, chromogenic bacteria may fail to produce pigment, *e g ,* under anaerobic conditions, *B. pyocyaneus* does not produce its pigment; many conditions interfere with pigment production; occasionally a part

of a culture will show pigment while other parts fail to show any. At present the cause of these variations is not well understood.

3. To demonstrate pigment production, inoculate the surface of various solid media and incubate under following conditions: At 37° C. and at 20° C., aerobically, exposed and unexposed to light.

4. Spreading a portion of the growth on white glazed paper brings out colors more sharply.

Liquefaction of Gelatin and Coagulated Blood Serum.—1. The liquefaction of gelatin and coagulated blood serum (Löffler's) is due to the production of a proteolytic enzyme by certain bacteria.

2. The demonstration of the production of this enzyme is of value in the identification of bacteria. It is to be kept in mind, however, that there may be considerable difference in production of this ferment by different cultures of the same variety of bacterium.

3. To demonstrate this proteolytic activity of bacteria, inoculate a tube of liquid gelatin; shake well to distribute organisms, and after incubating at 37° C. for twenty-four to forty-eight hours, place in ice chest or in glass of ice water. If digestion has taken place the medium will remain liquid while a sterile control will solidify. Some organisms liquefy slowly; therefore incubation should continue for at least seven days when liquefaction cannot be demonstrated earlier.

Coagulation of Milk.—Certain bacteria when grown in milk cause its coagulation. In most cases this is due to formation of acid as the result of fermentation of the lactose present. A rennin-like enzyme has been found to be produced by some bacteria which is capable of coagulating the milk independent of the acids.

To demonstrate this property of bacteria, inoculate a culture of plain milk. Incubate and examine daily. Coagulation usually occurs within one to seven days with whey formation.

Formation of Acid and Gas from Carbohydrates.—1. Acid and gas may both be produced by many bacteria in culture media containing sugars or higher alcohols. Some, however, produce only acid, in which case the acid formed is usually pure lactic or acetic acid.

2. To demonstrate the formation of acid, inoculate a medium containing a carbohydrate or higher alcohol and an indicator. The medium should be neutral or slightly alkaline in reaction. Incubate, and if acid is formed the color of the indicator will change accordingly; if litmus is used, the change will be from blue to red.

3. Another method is to determine by accurate titration the reaction of the medium before and after bacterial growth.

4. To demonstrate gas, the medium should be such that the gas formed will not escape but collect as visible bubbles in the medium, or the medium may be placed in fermentation tubes so the gas formed will collect in the closed arm.

5. Media commonly used for demonstrating acid or gas formations are: litmus milk; Hiss's serum water media containing carbohydrates; Russel's double sugar; glucose or other sugar broths in fermentation tubes, etc.

Indole Production.—Certain bacteria, particularly those belonging to the colon and proteus groups, produce indole by decomposition of protein. The recognition of this substance in cultures may be used as a means of differentiating certain closely related organisms. The technic is as follows:

1. Inoculate peptone solution or plain broth and incubate two to seven days.

2. Add 6 or 8 drops of concentrated sulphuric acid to the culture.

3. If both indole and a nitroso body are present, a violet pink coloration will be produced. This is the *cholera red* reaction.

4. Should no change take place, add 1 c.c. of a 1 : 10,000 dilution of sodium nitrate.

5. If indole is present, a pink color will be produced.

6. By using trypsinized broth (Rivas) the culture may be examined in less than forty-eight hours.

Ehrlich's Test for Indole.—1. Inoculate tubes of Dunham's peptone solution with organisms to be tested. As controls inoculate one tube with an organism known to produce indole (*B. coli communis*) and one tube with an organism known not to produce indole (*B. typhosus*).

2. Incubate cultures for from four to six days.

3. Add to each tube 1 c.c. of ether; shake well and allow to stand until ether rises to the surface.

4. Overlay with a few drops of Ehrlich's reagent.

<div align="center">EHRLICH'S REAGENT</div>

Paradimethylaminobenzaldehyde .. .. ..   2 gm.
Alcohol (absolute) .   ... 190 c.c.
Hydrochloric acid (conc.) .   ...   40 c c.

5. A positive reaction is indicated by the development of a pink color at the point of contact between the ether and the reagent.

Vanillin Test for Indole.—1. To 5 c.c. of culture to be tested, add 5 drops of a 5 per cent alcoholic solution of vanillin. Also include controls recommended above.

2. Next add 2 c.c. of concentrated sulphuric acid.

3. If indole is present, an orange color will develop in two to three minutes. If tryptophan is present, a reddish-violet color will develop.

Voges-Proskauer Reaction.—1. Inoculate fermentation tubes containing 2 per cent glucose broth.

2. Incubate three days.

3. Add to the culture 1 c.c. of a 50 per cent solution of caustic potash solution and allow to stand at room temperature for twenty-four hours.

4. A positive reaction is indicated by the development of a pink color on

exposure to the air, due to the presence of acetyl-methyl carbinol. This test is of some value in differentiating closely related organisms.

5. Boiling 1 to 4 c.c. of the culture with 5 c.c. of a 10 per cent solution of sodium hydroxide is a rapid method.

6 Shaking or blowing into the tube to promote oxidation hastens the reaction.

Reduction of Nitrates.—Some bacteria are capable of reducing certain substances. A typical example is the changing of nitrates to nitrites The following test may be used to demonstrate this property·

1. Grow organism in broth containing nitrate for four or five days

2. To 4 c c. of the culture add 2 c c. of the reagent.

<div align="center">REAGENT</div>

<div align="center">Solution No. 1</div>

Naphthylamine	0 1 gm.
Water (distilled)	20 0 c c
Acetic acid (25 per cent)	105 0 c c

Dissolve the naphthylamine in the distilled water by heat Allow to cool, then filter and add the acetic acid

<div align="center">Solution No. 2</div>

Sulphanilic acid	0 5 gm.
Acetic acid (dil 1 : 16)	105 0 c c

Mix equal parts of solutions Nos 1 and 2 to obtain reagent for use in test These solutions should be kept separate, and sufficient mixed as needed

3. Place the mixture in warm water for fifteen or twenty minutes.

4 A positive reaction is indicated by the development of a pink color.

Sulphurated Hydrogen.—1. Cultivate the organism in Dunham's peptone water for forty-eight to seventy-two hours.

2. To 10 c c. of culture add 0.1 c.c. of a 1 per cent solution of lead acetate or ferric tartrate.

3 If the precipitate which forms turns black, sulphurated hydrogen is present.

Toxin Production.—1. The hemotoxins of streptococci, staphylococci, etc , are best demonstrated by cultivating the organism on Petri plates or slants of blood agar. The development of clear zones of hemolysis about the colonies denotes the production of hemotoxins or hemolysins.

2. A greenish discoloration of the medium about the colony is due to the transformation of hemoglobin into methemoglobin without the production of hemolysis.

3. Toxins are also detected by the Mandler or Berkefeld filtration of broth cultures and the injection of the sterile filtrates into guinea-pigs or other animals.

EXAMINING THE SKIN FOR PARASITES

Benbrook's Method.—A simple and rapid method for detecting skin mites is carried out by means of a scraping in paraffin oil by the method of Benbrook:
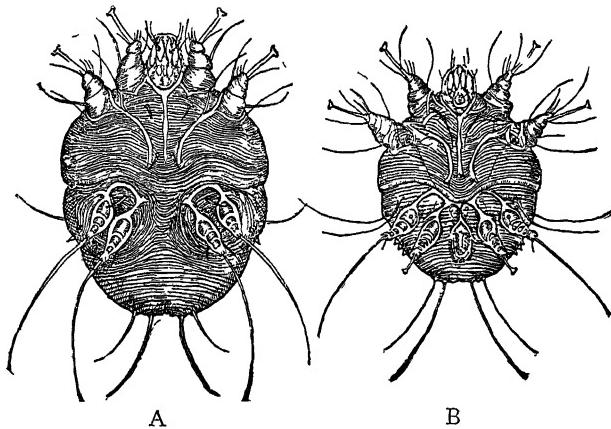
FIG 195—ACARUS SCABIEI (VENTRAL SURFACE)
A. Female   B Male   × 100   (From Schamberg, *Diseases of the Skin and Eruptive Fevers,* W. B. Saunders Co)

1. Sterilize a scalpel or other scraper in an alcohol or gas flame. Cool by dipping into water. Dry.

2. Place a drop of paraffin oil in the center of a microscope slide

3. Dip the scalpel in the paraffin oil drop (an oily scraper will pick up a specimen more easily than will a dry scraper)

4. Pinch a fold of skin showing lesions, between the thumb and forefinger, and scrape the crest of the fold with oily scalpel blade until lymph begins to ooze. Avoid drawing gross blood.

5. Transfer the scraping from the scalpel to the drop of oil on the slide.

6. Apply a micro cover glass to the drop with the aid of forceps.

7. Systematically examine the material under the cover glass, using the low power of the microscope and rather low illumi-

FIG 196—FEMALE GRAIN ITCH MITE
× 300.
(From Schamberg, *Diseases of the Skin and Eruptive Fevers,* W B Saunders Co)

nation. The oil renders the skin scales transparent and parasites appear rather prominently. Mites may live for several days in such a preparation. In some cases, several scrapings may be necessary in order to find mites.

8. Certain of the larger mites may be seen upon gross examination by scraping the lesions with a dry scalpel or knife blade and placing the scraping upon a piece of black paper or cloth exposed to sunlight and warmth. The mites may be seen as tiny white dots moving about. This method, of course, cannot be depended upon for an accurate diagnosis.
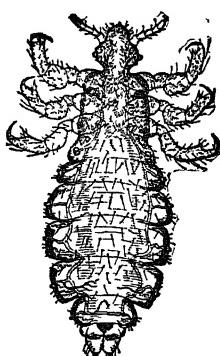
FIG 197.—PEDICULUS CAPITIS   FIG 198.—PEDICULUS CORPORIS.   FIG 199.—PEDICULUS PUBIS.
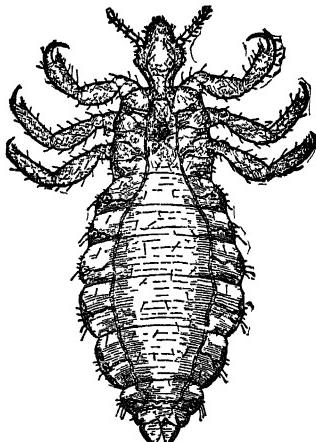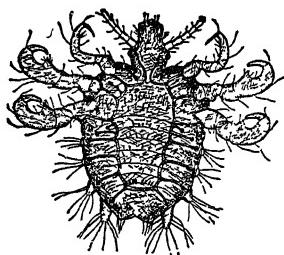
(From Schamberg, *Diseases of the Skin and Eruptive Fevers,* W. B. Saunders Co)

Parasites.—The following parasites are of particular importance in diseases of human beings:

1. The *Acarus scabiei,* which produces scabies (Fig. 195)
2. The grain or straw itch mite, which produces acarodermatitis urticarioides (Schamberg) (Fig. 196)
3. The *pediculi* (Figs. 197, 198 and 199)

CHAPTER XIX

DIAGNOSTIC BACTERIOLOGICAL METHODS

REGIONAL DISTRIBUTION OF PATHOGENIC ORGANISMS

1. When material is submitted for bacteriological examination its source should be stated, as this information may indicate the probable bacteriological findings and guide the method of examination; for example, pus from an acute furuncle or "boil" usually shows a staphylococcus in pure culture. It is therefore helpful in bacteriological diagnosis to keep in mind the regional distribution of the pathogenic organisms, although in smears and cultures of the skin and mucous membranes open to air contamination, various non-pathogenic bacteria may be encountered which are not included herewith except such common ones as *B. subtilis, B. proteus-vulgaris* and the like.

2. No attempt has been made to include all the organisms found and described in the different locations listed. For example, at least forty-six different ones have been found in the saliva and even a larger number in the feces, but the majority of them are of little or no importance. For the identification of these the worker is referred to the third edition of Bergey's *Manual of Determinative Bacteriology.*

3. When material like feces containing many different bacteria is submitted for bacteriological examination, the specimen should be accompanied by a request designating the particular organism or organisms to be examined for.

4. Since a smear of material or its culture stained by the Gram method at once yields very useful information, it has proven helpful to arrange the following regional distribution on the basis of this differential stain.

5. Animal parasites (protozoa and metazoa) are not included but are given elsewhere in the sections devoted to the examination of feces, sputum, etc.

6. The new nomenclature of the American Society of Bacteriologists is given as well as the older names for the various microorganisms for the assistance this may give in gaining familiarity with the newer terminology.

7. A few of the pathogenic microorganisms may be identified by stained smears alone (gonococci, diphtheria bacilli, tubercle bacilli, etc.) but the majority require a study of cultures and various biological characteristics described on succeeding pages.

8. It is hoped that the outlines here given will prove particularly helpful to inexperienced bacteriologists as indicating the kinds of bacteria to be kept in mind or especially looked for according to the source of the material.

Bacteriology and Mycology of the Skin

Gram-positive
- Staphylococci
- Streptococci including *Streptococcus scarlatinae* and *Streptococcus erysipelatis*
- Pneumococci
- B. diphtheriae *(Corynebacterium diphtheriae)*
- B. pseudodiphtheriae *(Corynebacterium pseudodiphtheriae)*
- B. acnes *(Corynebacterium acnes)*
- B. anthracis
- B. subtilis
- Erysipelothrix *(Erysipelothrix rhusiopathiae)*
- Actinomyces { *Actinomyces hominis* / *Actinomyces madurae* }
- Sporotrichia *(Sporotrichum beurmanni)*
- Blastomycetes
- *Tinea trichophytina* { *Microsporon audouini* / *Trichophyton* / *Achorion schoenleinii* / *Microsporon furfur* / *Microsporon minutissimum* }

Gram-negative
- B. pyocyaneus *(Pseudomonas aeruginosa)*
- B. mallei *(Pfeifferella mallei)*
- B. coli *(Escherichia coli)*
- B. rhinoscleromatis *(Klebsiella rhinoscleromatis)*
- M. catarrhalis *(Neisseria catarrhalis)*
- B. proteus vulgaris
- *Spirochaeta pertenuis (Treponema pertenue)*
- *Spirochaeta pallida (Treponema pallidum)*
- *Spirochaeta refringens (Treponema refringens)*

Acid-fast
- B. tuberculosis *(Mycobacterium tuberculosis)*
- B. leprae *(Mycobacterium leprae)*
- B. smegmatis *(Mycobacterium smegmatis)*

Bacteriology of Furuncles, Carbuncles, Cellulitis and Lymphadenitis

Gram-positive
- *Staphylococcus aureus* and *albus*
- *Streptococcus pyogenes*
- *B. anthracis*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. subtilis*

Gram-negative
- Gonococcus *(Neisseria gonorrhoeae)*
- *B. proteus vulgaris*
- Ducrey's bacillus (hemophilus of Ducrey)
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B. tularense (Pasteurella tularensis)*
- *B. pestis (Pasteurella pestis)*
- *Spirochaeta pallida (Treponema pallidum)*

Bacteriology and Mycology of Infected Wounds, Sinuses and Ulcers

Gram-positive	*Staphylococcus aureus* and *albus* *Streptococcus pyogenes* Pneumococcus *(Diplococcus pneumoniae)* *B. tetani (Clostridium tetani)* *B welchii (B. aerogenes-capsulatus)* *B. oedematis-maligni (Clostridium oedematis-maligni* or *Vibrion septique)* *B. oedematiens (Clostridium oedematiens)* *B. bellonensis* *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)* *B. anthracis* *B. subtilis* *Spirochaeta vincenti (Borrelia vincenti)* Actinomyces { *Actinomyces hominis* / *Actinomyces madurae* }
Gram-negative	*Erysipelothrix (Erysipelothrix rhusiopathiae)* *Sporotrichia (Sporotrichum beurmanni)* Blastomycetes *B. fusiformis (Fusiformis dentium)* *B. pyocyaneus (Pseudomonas aeruginosa)* *B. coli (Escherichia coli)* *B. proteus-vulgaris* Actinobacillus *(Actinobacillus lignieresi Brumpt)*

Bacteriology and Mycology of the Eye (Lids, Conjunctiva, Cornea and Lacrimal Ducts)

Gram-positive	*Staphylococcus aureus* and *albus* Streptococci Pneumococci *M. tetragenus (Gaffkya tetragena)* *B. diphtheriae (Corynebacterium diphtheriae)* *B. xerosis (Corynebacterium xerosis)* *B. anthracis* *B. subtilis* *Oïdium albicans* Leptothrices Streptothrices *Actinomyces hominis* *Aspergillus fumigatus*
Gram-negative	Gonococcus *(Neisseria gonorrhoeae)* *M. catarrhalis (Neisseria catarrhalis)* Meningococcus *(Neisseria intracellularis)* Bacillus of Morax-Axenfeld *(Hemophilus lacunatus)* Bacillus of Koch-Weeks *(Hemophilus conjunctivitidis)* *B. influenzae (Hemophilus influenzae)* *B. pyocyaneus (Pseudomonas aeruginosa)* *B. tularensis (Pasteurella tularensis)* *B. nedden* *Spirochaeta pallida (Treponema pallidum)*
Acid-fast	*B. tuberculosis (Mycobacterium tuberculosis)* *B. leprae (Mycobacterium leprae)*

Bacteriology of the Nose and Accessory Sinuses

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *B. segmentosus (Corynebacterium segmentosum)*
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. subtilis*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- Meningococcus *(Neisseria intracellularis)*
- *B. mucosus ozaenae (Klebsiella ozaenae)*
- *B rhinoscleroma (Klebsiella rhinoscleromatis)*
- *B. ozaenae (Klebsiella ozaenae)*
- Friedlander's bacillus *(Klebsiella pneumoniae)*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B influenzae (Hemophilus influenzae)*
- *B. fusiformis (Fusiformis dentium)*
- *B. pertussis (Hemophilus pertussis)*
- *B. mallei (Pfeifferella mallei)*
- *B proteus vulgaris*
- *Spirochaeta vincenti (Borrelia vincenti)*
- *Spirochaeta pallida (Treponema pallidum)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*
- *B. leprae (Mycobacterium leprae)*

Bacteriology of the Throat (Including Nasopharynx and Tonsils)

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. subtilis*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- *M. flavus (Diplococcus perflava)*
- *M pharyngis siccus (Diplococcus siccus)*
- Meningococcus *(Neisseria intracellularis)*
- *B. influenzae (Hemophilus influenzae)*
- *B. fusiformis (Fusiformis dentium)*
- *B. pertussis (Hemophilus pertussis)*
- *B pyocyaneus (Pseudomonas aeruginosa)*
- *B. proteus vulgaris*
- Friedländer's bacillus *(Klebsiella pneumoniae)*
- *Spirochaeta vincenti (Borrelia vincenti)*
- *Spirochaeta pallida (Treponema pallidum)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*
- *B. leprae (Mycobacterium leprae)*

Bacteriology of the Ears and Mastoid

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. subtilis*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- Meningococcus *(Neisseria intracellularis)*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B. influenzae (Hemophilus influenzae)*
- *B. fusiformis (Fusiformis dentium)*
- Friedlander's bacillus *(Klebsiella pneumoniae)*
- *B. proteus vulgaris*
- *Spirochaeta vincenti (Borrelia vincenti)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*

Bacteriology and Mycology of the Mouth (Including Gums and Saliva)

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *M. tetragenus (Gaffkya tetragena)*
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. acidophilus (Lactobacillus acidophilus)*
- *Leptotrichia buccalis*
- Streptothrices
- *Oïdium albicans*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- *B. fusiformis (Fusiformis dentium)*
- *B. coli (Escherichia coli)*
- *B. influenzae (Hemophilus influenzae)*
- *Vibrio sputigenus*
- *Spirochaeta vincenti (Borrelia vincenti)*
- *Spirochaeta pallida (Treponema pallidum)*
- *Spirochaeta microdentium (Treponema microdentium)*
- *Spirochaeta macrodentium (Treponema macrodentium)*
- *Spirochaeta mucosum (Treponema mucosum)*

Bacteriology of the Teeth (Dentine and Periapical Infections)

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. acidophilus (Lactobacillus acidophilus)*
- *B. mesentericus ruber (B. teres)*
- *B. mesentericus-vulgatus (B. graveolens)*
- *B. mesentericus-fuscus (B. mesentericus)*
- *B. subtilis*
- Various sarcinae

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- *B. proteus-vulgaris (Proteus zenkeri)*

Bacteriology and Mycology of Sputum (Including Secretions Secured by Bronchoscopic Drainage)

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *M. tetragenus (Gaffkya tetragena)*
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- Blastomycetes
- Actinomyces { *Actinomyces hominis* / *Actinomyces asteroides* }
- Streptothrices
- *Leptotrichia buccalis*
- *Oïdium albicans*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- Meningococcus *(Neisseria intracellularis)*
- Friedlander's bacillus *(Klebsiella pneumoniae)*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B. fusiformis (Fusiformis dentium)*
- *B. pertussis (Hemophilus pertussis)*
- *B. influenzae (Hemophilus influenzae)*
- *B. proteus vulgaris*
- *B. coli (Escherichia coli)*
- *Spirochaeta vincenti (Borrelia vincenti)*
- *Spirochaeta microdentium (Treponema microdentium)*
- *Spirochaeta macrodentium (Treponema macrodentium)*
- *Spirochaeta mucosum (Treponema mucosum)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*

Bacteriology of the Cerebrospinal Fluid

Gram-positive
- Streptococci
- Pneumococci
- Staphylococci
- *M. tetragenus (Gaffkya tetragena)*
- Actinomyces
- Yeasts

Gram-negative
- Meningococcus *(Neisseria intracellularis)*
- *B. influenzae (Hemophilus influenzae)*
- *B. coli (Escherichia coli)*
- *B. typhosus (Eberthella typhi)*
- *B. pestis (Pasteurella pestis)*
- *B. mallei (Pfeifferella mallei)*
- *Spirochaeta pallida (Treponema pallidum)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*

Bacteriology of Pleural and Pericardial Fluids

Gram-positive
- Staphylococci
- Streptococci
- Pneumococci
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. subtilis*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- Friedlander's bacillus *(Klebsiella pneumoniae)*
- *B. influenzae (Hemophilus influenzae)*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B. proteus-vulgaris*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*

Bacteriology of Peritoneal Fluid

Gram-positive
- Staphylococci
- Streptococci
- Pneumococci
- *B. subtilis*

Gram-negative
- *B. coli (Escherichia coli)*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B. proteus-vulgaris*
- *B. typhosus (Eberthella typhi)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*

Bacteriology and Mycology of Bile (Duodenal Drainage)

Gram-positive
- Staphylococci
- Streptococci
- *M. tetragenus (Gaffkya tetragena)*
- *B pseudodiphtheriae (Corynebacterium pseudodiph-theriae)*
- *B. subtilis*
- *Saccharomyces cerevisiae*, etc.

Gram-negative
- *B. coli (Escherichia coli)*
- *B. typhosus (Eberthella typhi)*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- Friedländer's bacillus *(Klebsiella pneumoniae)*
- *B. proteus-vulgaris*

Bacteriology of Feces and the Rectum

Gram-positive
- Staphylococci
- Streptococci (including Bargen's coccobacillus)
- Pneumococci
- *B. welchii* ⎫
- *B. tetani* ⎭ and other anaerobes
- *B. acuminatus (Bacteroides acuminatus)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiph-theriae)*
- *B. anthracis*
- *B. subtilis*
- *B acidophilus* and lactic acid groups
- *B. bifudus (Bacteroides bifudus)*
- *Monilia psilosis*

Gram-negative
- Gonococcus *(Neisseria gonorrhoeae)*
- *M. catarrhalis (Neisseria catarrhalis)*
- *B. coli (Escherichia coli)*
- *B. cholerae (Vibrio comma)*
- *B. typhosus (Eberthella typhi)*
- *B. paratyphosus A (Salmonella paratyphi)*
- *B. paratyphosus B (Salmonella schottmuelleri)*
- *B. enteritidis (Salmonella enteritidis)*
- *B. dysenteriae (Shigella dysenteriae)*
- *B. faecalis-alcaligenes*
- *B ambiguus (Shigella ambigua)*
- *B. liquefaciens (Bacteroides liquefaciens)*
- *B. proteus vulgaris*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*
- *B. leprae (Mycobacterium leprae)*

Bacteriology of the Urine (from Bladder and by Ureteral Catheterization)

Gram-positive
- Staphylococci
- Streptococci
- *B. subtilis*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*

Gram-negative
- Gonococcus *(Neisseria gonorrhoeae)*
- *M. catarrhalis (Neisseria catarrhalis)*
- Meningococcus *(Neisseria intracellularis)*
- *B. proteus-vulgaris*
- *B. pyocyaneus (Pseudomonas aeruginosa)*
- *B. typhosus (Eberthella typhi)*
- *B. paratyphosus A (Salmonella paratyphi)*
- *B. paratyphosus B (Salmonella schottmuelleri)*
- *B. coli (Escherichia coli)*
- *B. dysenteriae (Shigella dysenteriae)*
- *B. abortus (Alcaligenes, or Brucella, abortus)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*
- *B. smegmatis (Mycobacterium smegmatis)*

Bacteriology of the Urogenital organs (Including the Prostate Gland)

Gram-positive
- Staphylococci
- Streptococci
- Pneumococci
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. subtilis*

Gram-negative
- Gonococcus *(Neisseria gonorrhoeae)*
- *M. catarrhalis (Neisseria catarrhalis)*
- *B. proteus-vulgaris*
- Bacillus of Ducrey (hemophilus of Ducrey)
- *B. coli (Escherichia coli)*
- *B. acidophilus (Lactobacillus acidophilus)*
- *Spirochaeta pallida (Treponema pallidum)*
- *Spirochaeta refringens (Borrelia refringens)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*
- *B. smegmatis (Mycobacterium smegmatis)*

Bacteriology of the Blood

Gram-positive
{
Staphylococci
Streptococci
Pneumococci
B. anthracis
B. welchii, etc.
}

Gram-negative
{
Meningococcus *(Neisseria intracellularis)*
Gonococcus *(Neisseria gonorrhoeae)*
B. abortus *(Alcaligenes,* or *Brucella, abortus)*
B typhosus *(Eberthella typhi)*
B. paratyphosus A *(Salmonella paratyphi)*
B. paratyphosus B *(Salmonella schottmuelleri)*
B dysenteriae *(Shigella dysenteriae)*
B. coli *(Escherichia coli)*
B pyocyaneus *(Pseudomonas aeruginosa)*
Friedlander's bacillus *(Klebsiella pneumoniae)*
B. influenzae *(Hemophilus influenzae)*
B. pestis *(Pasteurella pestis)*
Spirochaeta pallida *(Treponema pallidum)*
Spirochaeta recurrentis *(Borrelia recurrentis)*
}

Acid-fast
{
B. tuberculosis *(Mycobacterium tuberculosis)*
B. leprae *(Mycobacterium leprae)*
}

BACTERIOLOGICAL DIAGNOSIS OF STAPHYLOCOCCUS INFECTIONS

1. Make a thin smear of material to be examined on a slide and stain by the Gram method.

2. Inoculate culture media. If material is pus from an abscess, the organisms are likely to be in pure culture and slants may be used. If other organisms are likely to be present, inoculate plates. Plain or blood agar may be employed; the latter is preferred in order to determine if hemolysis occurs.

3. Incubate for twenty-four hours. Examine colonies. Stain by Gram.

4 Staphylococci occur singly, in pairs, and in irregular groups (Fig. 200); rarely in packets.

5. They are gram-positive, but gram-negative cocci may be seen. Growth is fair to good on ordinary media. Gelatin is commonly liquefied. As a rule, carbohydrates are
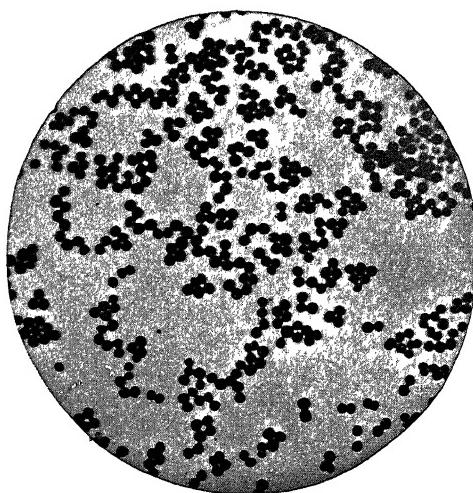
Fig 200—Staphylococcus Pyogenes Aureus
(After Gunther)

fermented with the production of acid.   Hemolysis commonly occurs.   Pigment is white or orange, or less commonly lemon-yellow.

<div align="center">KEY TO IDENTIFICATION (BERGEY)</div>

I. Orange pigment

    1 Lactose fermented.   Gelatin liquefied

        1. *Staphylococcus aureus*

II. Lemon-yellow pigment

    1. Lactose fermented.   Gelatin liquefied

        2. *Staphylococcus citreus*

III. White or colorless growth on solid media

    1. Lactose fermented.   Gelatin liquefied

      (a) Ferment sucrose but not mannitol or raffinose

        3 *Staphylococcus epidermidis*

      (b) Ferment sucrose and mannitol but not raffinose

        4. *Staphylococcus albus*

      (c) Ferment sucrose, mannitol and raffinose

        5. *Staphylococcus pharyngis*

6. The two varieties usually encountered are *Staphylococcus albus* and *Staphylococcus aureus*

The aureus is found about twice as frequently in pathological processes. The albus variety is the common cause of stitch abscess; it is less virulent than the aureus and seldom produces pyemia.   Both varieties may be present in the same infection.

Staphylococcus albus and *aureus* are normally present in the skin and mucous membranes.

The mere presence of the staphylococcus in cultures from a lesion does not by any means indicate that it is the cause of the infection.   As noted above, they are normally present in the skin and mucous membranes and may contaminate cultures, including blood cultures.

BACTERIOLOGICAL DIAGNOSIS OF STREPTOCOCCUS INFECTIONS

1. Make thin smear of exudate to be examined on a cover glass or slide.

2. Fix and stain by Gram's method

3. Prepare cultures.   Blood agar is the medium of choice; glucose-hormone broth and brain broth are excellent.   See page 259 for technic of making blood cultures.

4. Slants may be inoculated when plates are not available.   The slant or broth cultures should be sent immediately to the laboratory for transference to plates.

5. After incubating plates twenty-four to forty-eight hours, examine.

FIG. 201.—STREPTOCOCCUS PYOGENES.   (Zinsser.)

Organism	Blood Agar	Lactose	Mannitol	Salicin
Streptococcus pyogenes..........	Hemolytic	+	−	+
Streptococcus scarlatinae........	Hemolytic	+	−	+
Streptococcus puerperalis........	Hemolytic	+	−	+
Streptococcus erysipelatis........	Hemolytic	+	−	+
Streptococcus anginosus.........	Hemolytic	+	−	−
Streptococcus equi..............	Hemolytic	−	−	+
Streptococcus mitior............	Viridans	+	−	+
Streptococcus salivarius..........	Viridans	+	−	+
Streptococcus faecalis...........	Viridans	+	+	+
Streptococcus equinus...........	Viridans	−	−	+
Streptococcus nonhaemolyticus...	Nonhemolytic	+	−	+
Streptococcus cardio-arthritidis...	Nonhemolytic	+ or −	−	+

6. Occurs in pairs, short or long chains, never in packets (Fig. 201). Generally gram-positive. Capsules rarely formed. Grows as effused, translucent, often small isolated colonies on agar streak. In stab cultures little surface growth is developed. Many carbohydrates are fermented with formation of acid, but inulin is rarely attacked. Generally fail to liquefy gelatin or reduce nitrates. Some species hemolyze blood (*Streptococcus haemolyticus*), producing zones of hemolysis around colonies (Fig. 202); others produce methemoglobin (*Streptococcus viridans*), while a smaller number are without action on blood (*Streptococcus nonhaemolyticus*).

FIG. 202.—ZONES OF HEMOLYSIS AROUND COLONIES OF HEMOLYTIC STREPTOCOCCI.

(From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

7. Type diagnosis is made according to (*a*) source of material; (*b*) action on blood agar, and (*c*) whether or not acid is produced according to the table on page 338.

8. The pneumococcus produces small greenish colonies not unlike *Streptococcus viridans*, and at times it may be difficult to differentiate these two organisms without special tests.

BACTERIOLOGICAL DIAGNOSIS OF PNEUMOCOCCUS INFECTIONS

1. Choose for examination a portion of the more tenacious and blood-tinged sputum and spread in thin smear on slide. Spinal or other fluids (pleural, etc.) should be centrifuged and smears made from sediment unless they are purulent.

2. Dry in air and fix with gentle heat.

3. Stain by Gram's method. The gram-positive pneumococci will be recognized as dark violet or purple lancet-shaped diplococci; the gram-negative organisms will be colored a faint pink or red. Sometimes with the Gram stain, capsules will be seen surrounding the pneumococci; these are better stained by the Huntoon method (Fig. 203).

4. With platinum loop streak out one or two blood agar plates with material to be examined.

FIG. 203.—PNEUMOCOCCI WITH STAINED CAPSULES. (Zinsser.)

5. Place plates in incubator for twenty-four or forty-eight hours.

6. Examine characteristic colonies for gram-positive diplococci.

The pneumococcus colony on blood agar is small, flat and greenish with no hemolysis. It may be confused with the *Streptococcus viridans* as the colo-

nies are very similar and it often forms small chains.   Bouillon cultures of pneumococci are bile soluble, streptococci are not.   The pneumococci ferment inulin; streptococci do not.

Bile Solubility Test.—1. To a twenty-four-hour bouillon culture of organisms to be tested, add o 5 to 1.0 c c. of ox bile which has been filtered and sterilized (to 10 c c. of bouillon add 1 c c. of bile).

2. Place the cultures in incubator for one half hour.

3. Streptococcus cultures will remain unchanged.   Pneumococcus cultures become clear (organism is bile soluble).

Fermentation of Inulin.—1. Inoculate Hiss's serum water medium to which inulin has been added.   Incubate twenty-four to forty-eight hours.

2. Pneumococci will ferment inulin with the formation of acid (red). Streptococci do not ferment inulin (no change in color).

Types of Pneumococci.—There are three types of pneumococci, desig-
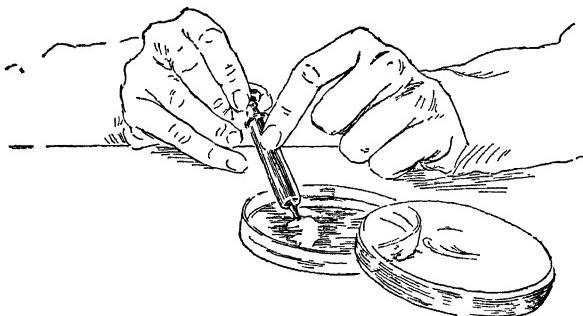
FIG. 204.—WASHING SPUTUM IN SALT SOLUTION
(From Wadsworth, *Standard Methods,* Waverly Press, Inc, Baltimore)

nated as Types I, II and III, and a heterogeneous group comprising at least ten or more types designated as Group IV.   They differ in virulence and immunological reactions.   Strains belonging to Group IV are the least virulent and are the ones commonly found in normal throats and are called the "mouth strains."   The most virulent are Types II and III; the latter has a tendency to grow in chains and develops a very large capsule and at one time was known as the *Streptococcus mucosus.*

Agglutination Test for Type Differentiation.—1. If the sputum is very fluid and has no firm portions, inoculate the mouse directly.   Otherwise, take up a firm portion, consisting of not more than o 5 c c., in a sterile glass syringe without a needle and wash it three times in sterile salt solution.   Grind the washed sputum in a sterile mortar, adding from 1.5 to 2.5 c.c. of broth during the process (Figs. 204 and 205).

2. Inoculate a mouse intraperitoneally with from 0.5 c.c. to 1 c.c. of the diluted, washed sputum.

3. Within from four to eight hours, puncture the peritoneum of the mouse with a sterile needle attached to a syringe and withdraw one or two drops of

exudate. Spread this on a slide and stain by Gram's method. If microscopic examination shows as many gram-positive cocci as may be found in an eighteen-hour broth culture, chloroform the mouse and proceed with the test.

If only a few organisms are found, leave the mouse until the following morning, and if it is not dead then, make a second exploratory puncture. If the stained preparations made at this time do not show sufficient growth, leave the mouse, and report on the results of Avery's cultural method, or on the growth on the plates inoculated with the sputum.

4. Since it is desired to recover the organisms in pure culture, carefully observe sterile precautions throughout the autopsy.

5. When the skin has been laid back, make a short, longitudinal opening in the abdominal wall (Fig. 206). Take a loopful of the peritoneal exudate and streak half a blood agar plate. Also make a smear for Gram's stain. Then enlarge the opening in the abdominal wall and note if the exudate is
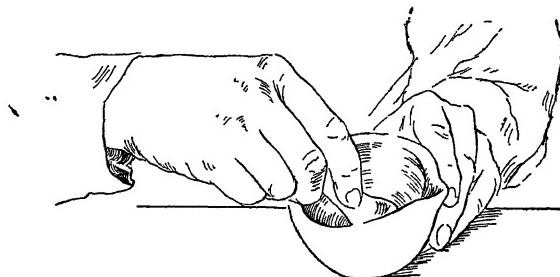
FIG. 205 —EMULSIFYING SPUTUM IN BROTH BEFORE INOCULATION OF MOUSE. (From Wadsworth, *Standard Methods,* Waverly Press, Inc , Baltimore.)

sticky, suggesting the presence of Type III pneumococci or *B. mucosus-capsulatus* (Friedlander's bacillus). Using a bulb pipet, wash the peritoneum thoroughly with from 3 to 4 c.c. of salt solution, and put the washings in a centrifuge tube. Then, with sterile instruments, open the thoracic cavity, and, from the heart's blood, inoculate a tube of pneumococcus broth and streak the other half of the blood agar plate which has been used for the peritoneal exudate (Fig. 207).

6. Centrifugalize the peritoneal washings at low speed for a few minutes; pour the supernatant suspension of organisms into a second centrifuge tube, and discard the sediment, which contains cellular débris from the peritoneum. Centrifugalize the suspension at high speed for from fifteen to twenty minutes or until it is perfectly clear. Remove with a pipet the supernatant fluid for a precipitation test (see below) and resuspend the sediment in salt solution for this agglutination test. Perform both tests as a matter of routine, since the former, in some instances, and the latter, in others, has been found to give more prompt and definite results.

7. Use for the routine tests Types I, II, and III antipneumococcus serum, and test, at the same time, the solubility of the organisms in ox bile. Be sure

the serum is clear each time before it is used, since the presence of a precipitate would obscure the reaction. If there is a precipitate present, centrifugalize the serum under aseptic precautions. Also be sure that the salt solution used in making the suspensions and serum dilutions is free from sediment.

8. Emulsify the sediment with normal salt solution, making a moderately heavy suspension (should equal at least an eighteen-hour bouillon culture of pneumococcus).

9. Place 0.5 c.c. of this emulsion into each of six small test tubes.

10. Into the first tube add 0.5 c.c. of Type I pneumococcus serum (un-
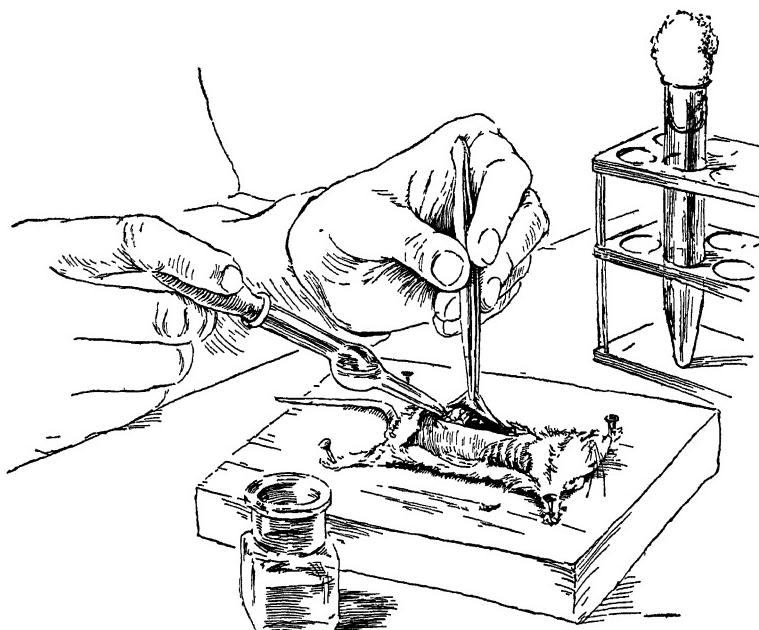
FIG 206.—COLLECTING PERITONEAL WASHINGS.
(From Wadsworth, *Standard Methods,* Waverly Press, Inc, Baltimore.)

diluted); to the second tube add Type II serum (undiluted); to the third tube add Type II serum (diluted 1:20); to the fourth tube add Type III serum (undiluted); to the fifth tube add a few drops of bile to determine if the emulsion contains chiefly bile-soluble organisms. To the sixth tube add 0.5 c.c. saline (control).

11. Mix well; place in water bath at 37° C. for one hour.

12. Agglutination is indicated by a flocculent appearance in contrast to uniform cloudiness of the control, along with rapid settling of the agglutinated organisms leaving the fluid above clear. The bile tube clears; if not, the suspension is probably not of pneumococci.

13. If agglutination takes place in the tube containing Type I serum and not in any of the other tubes, the strain is Type I; if agglutination occurs

in both the tubes containing Type II serum (undiluted and diluted), the strain is a typical Type II; if agglutination occurs only in the tube containing undiluted Type II serum, the strain is an atypical Type II; if agglutination occurs in the tube containing Type III serum, the strain is Type III. If no agglutination takes place in any of the tubes, the strain belongs to Group IV.

Whenever there is an indication of the presence of more than one type of pneumococcus, plate the culture and make an agglutination test with each subculture isolated, in an effort to isolate the types.

Contaminating organisms, especially streptococci, may cause irregular agglutination reactions, but they are not dissolved by the bile. Such cultures should be plated and the pneumococci, if present, obtained in pure culture.
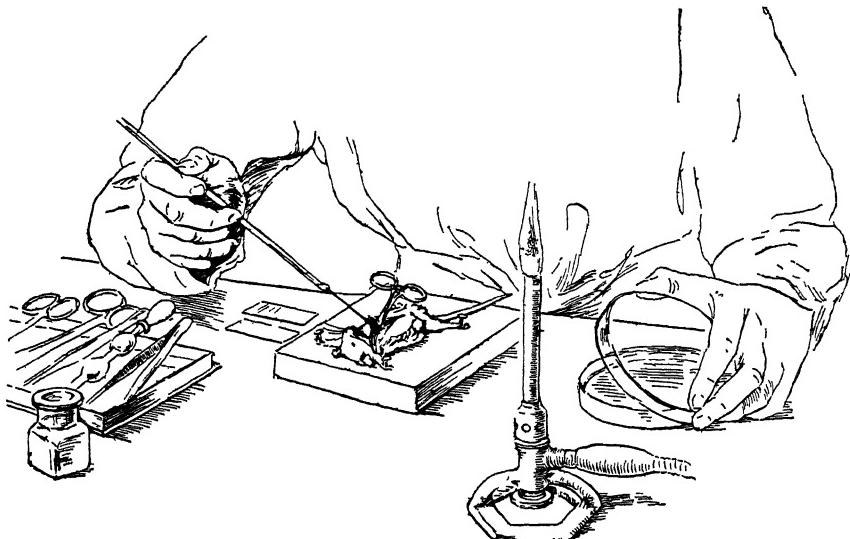
FIG. 207.—HEART BLOOD CULTURE.
(From Wadsworth, *Standard Methods,* Waverly Press, Inc., Baltimore.)

A precipitation test employing the supernatant peritoneal washing may be set up as follows in six small test tubes.

No. 1 : 0.3 c.c. serum Type I (undil.) +0.3 c.c. supernatant peritoneal washings
No. 2 : 0.3 c.c. serum Type I (dil. 1 :20)+0.3 c.c. supernatant peritoneal washings
No. 3 : 0.3 c.c. serum Type II (undil.) +0.3 c.c. supernatant peritoneal washings
No. 4 : 0.3 c.c. serum Type II (dil. 1 :20)+0.3 c.c. supernatant peritoneal washings
No. 5 : 0.3 c.c. serum Type III (undil.) +0.3 c.c. supernatant peritoneal washings
No. 6 : 0.3 c.c. serum Type III (dil. 1 .20)+0.3 c.c. supernatant peritoneal washings

Add the supernatant fluid slowly to each tube and look for the formation of a ring. Observe again at the end of fifteen minutes, heating in a water bath at 37° C. Then shake the tube gently and return them to the water bath.

After incubation a fibrin web may appear in all the tubes, and care must be taken not to confuse this with a specific reaction.

Precipitation Tests for Type Differentiation.—1. Transfer from 3 to 10 c.c. of sputum, depending on the amount available, to a 7 by 1 inch Pyrex test tube and immerse it in boiling water for several minutes or until a rather firm coagulum results.

2. Break this up with a glass rod or pipet and add sufficient o 85 per cent salt solution to make about 1 c.c. of fluid.

3. Immerse the tube in boiling water again for a few minutes, shaking it several times during the heating.

4 Centrifugalize the extract at high speed for from five to fifteen minutes and layer o 3 c c of the clear supernatant fluid on an equal amount of each of the three undiluted type antipneumococcus sera. To do this, tilt the agglutination tubes containing the sera, and allow the extract to flow slowly down the sides.

5. If the specimen is satisfactory for the test and contains Type I, II or III pneumococci, a ring of precipitate will usually be formed at the surface of contact of the two fluids in the tube containing the homologous serum

6. If the reaction does not occur immediately, plug the tubes loosely, without shaking, and incubate them at from 50° to 55° C. Observe after five minutes and again after fifteen minutes incubation. Then shake the tubes gently and reincubate for not more than one hour

7. After shaking, a definite clouding throughout, or a precipitation, will usually appear in the homologous serum. If definite precipitation is obtained by Krumwiede's method in any one of the type sera, it is not necessary to confirm the reaction by animal inoculation or cultural tests

Avery Cultural Precipitation Test.—1. Inoculate 4 c c of Avery's medium (meat infusion broth), containing 1 per cent glucose and 5 per cent sterile defibrinated blood, with 0.2 c.c. of diluted, washed sputum or with same amount of fluid sputum.

2. Mix and incubate for five to seven hours.

3 Make a smear and stain by Gram's method If a good growth of gram-positive diplococci has occurred, centrifugalize for a few minutes to throw down the blood cells.

4 Transfer 3 c.c of the supernatant fluid to a sterile centrifuge tube and add 1 c c. of sterile ox bile.

5. Incubate for thirty minutes to dissolve the pneumococci.

6. Centrifugalize at high speed for twenty minutes.

7. With the clear supernatant bile solution, set up the precipitation test described above for the mouse precipitation test.

Blood Cultures.—1. Centrifugalize 10 c c. of a blood culture, containing a pure growth of gram-positive cocci, at low speed to throw down the blood cells.

2. Make an agglutination test as described under the mouse method, using the supernatant broth culture

Spinal Fluid and Pleural Exudates.—1. Make a smear and stain by Gram's method.

2. If a large number of gram-positive diplococci are present, centrifugalize

briefly to throw down pus cells and conduct agglutination tests with the supernatant suspension of diplococci.

3. The balance of the fluid may be recentrifugalized at high speed for twenty minutes and the clear supernatant fluid used for a precipitation test.

4. If the diplococci are too few for these direct tests, inoculate a mouse with 0.5 c.c. and conduct tests with the peritoneal washings as described above.

5. Or inoculate 4 c c. of Avery's medium with 1 c.c. of fluid and conduct the test as described above.

BACTERIOLOGICAL DIAGNOSIS OF GONOCOCCUS (NEISSERIA GONORRHOEAE) INFECTIONS

Smear Method.—1. Make two thin smears of exudate.

2. Dry in air and fix with heat.

3. Stain one slide with Loffler's methylene blue and the other by Gram's method.

4. Wash, dry, and mount.

The gonococci will appear as biscuit- or coffee-bean-shaped diplococci. They are gram-negative and therefore stain red or pink with this method. Streptococci and staphylococci occurring in diplococcus formation are sometimes gram-negative and may resemble gonococci.

In the pre-acute stage before the exudate becomes profuse, the organisms may be extracellular but become intracellular (Fig. 208) during the acute stage when the exudate is at its height. At this stage it is common to find many organisms gathered within one leukocyte while other cells in the immediate neighborhood have none. Later, when the infection becomes more chronic, the organisms become less numerous and are extracellular. In gonococci conjunctivitis the organisms may occur in or upon epithelial cells.

Vaginal Washing Method.—1. Collect as described on page 262

2. Centrifuge at once and pour off the supernatant fluid.

FIG 208 — GONOCOCCI WITHIN PUS CELLS
(Zinsser)

3. Prepare smears of sediment and stain for gonococci as described above.

This method of examination is used in the diagnosis of infantile vaginitis and often yields better results than direct smears.

Examination of Urine for Gonococci.—1. Have patient present himself with full bladder.

2. Have ready two sterile bottles or centrifuge tubes

3. Allow patient to void part in one bottle or tube and part in the second. While urinating in second tube, press the patient's prostate with the finger.

The first bottle or tube will contain material from the anterior urethra and the second will likely contain secretions from the posterior urethra and prostate gland.

4. Centrifuge the urine collected at high speed for five minutes and examine the sediment for gonococci. In case the above method is negative, the secretions should be stimulated by massage or injections of silver nitrate. Gonococcus vaccine has been recommended as a means of provoking an increase of the number of gonococci in chronic cases.

Cultural Method (Park and Williams).—1. Add a small amount of rich sterile ascitic fluid to tube containing swab (if patient is at a distance from the laboratory, the ascitic fluid is sent in a separate tube).

2. Transfer the exudate to ascitic fluid with sterile swab. Mix well by stirring the fluid well with swab.

3 Withdraw swab from ascitic fluid and smear over surface of ascitic agar (1:4) plates containing glucose. (Smear in strokes radiating from center of plate.)

4. With platinum loop streak other plates of the same media with ascitic fluid suspension.

5. Incubate at 36° C. for twenty-four hours.

Look for gonococcus-like colonies; if present make smears and stain by Gram's method and examine for gonococcus-like organisms. Then make sub-cultures on ascitic, hydrocele or blood-streaked agar.

The colonies of gonococci on ascitic agar are small, translucent, finely granular with lobate margin, grayish-white with pearly opalescence by transmitted light. Frequent transplants are necessary to keep the culture alive. Differentiation from other gram-negative diplococci is based upon fermentation reactions as follows:

Organism	Dextrose	Maltose	Saccharose
Gonococcus	Acid	No acid	No acid
M catarrhalis	No acid	No acid	No acid
Meningococcus	Acid	Acid	No acid

Method of Interpreting and Reporting Results.—1. A *positive* report may be rendered when smears show large numbers of pus cells with gram-negative intracellular diplococci or typical cultures or both. Smears of urethral discharge from early cases and likewise from chronic cases (gleet) showing pus cells, many extracellular gram-negative diplococci and at least a few typical intracellular organisms, may be also reported as positive. Special care is required in reporting upon vaginal smears because other diplococci resembling gonococci are encountered more frequently than in the urethra; here cultures should be made, especially in medicolegal cases.

A positive report may also be rendered in smears showing 50 per cent

or more polymorphonuclear pus cells with many extracellular and occasional intracellular gram-negative diplococci of typical morphology.

2. A *suspicious* report may be rendered in cases with discharge when smears show many polymorphonuclear pus cells but no intracellular diplococci.

3. A *negative* report may be rendered when smears show only a few polymorphonuclear pus cells with no suspicious intracellular diplococci and no clinical evidences of disease. In any positive. doubtful, or suspicious case, smears should be made once a week until a negative is obtained to be followed by three more well-made smears at intervals of about three days before a final report of negative is rendered.

BACTERIOLOGICAL DIAGNOSIS OF MENINGOCOCCUS (NEISSERIA INTRACELLULARIS) INFECTIONS

Smears of Spinal Fluid.—Owing to the fact that meningococci often undergo rapid autolysis, the specimen should be examined as soon as possible after fluid has been taken from patient.

FIG 209—MENINGOCOCCUS IN SPINAL FLUID   (Zinsser)

1. Centrifuge the fluid at high speed. If the fluid is very cloudy, direct smears may be made without centrifugation.

2. Make thin smears from the sediment.

3. Stain with Gram's stain; stain a second slide with methylene blue.

4. The finding of gram-negative cocci, either intracellular or extracellular, is presumptive evidence of meningococcus infection (Fig. 209). However,

this should be confirmed by culture. Some cocci are more difficult to decolorize than others, especially intracellular ones.

Cultures of Spinal Fluid.—1. As soon as possible after the fluid is withdrawn from patient, place 1 c.c. on each of several blood agar or Loffler's blood serum plates or tubes and spread over surface. Glucose ascites agar neutral to phenolphthalein and hormone blood agar are also excellent media; the liver medium of Dopter is good for stock transplants.

2. Incubate at 37° C. for twenty-four hours.

3. If no growth occurs, allow to incubate another twenty-four hours.

4. Examine for meningococcus-like colonies, which are convex, slightly flattened, smooth, moist and shiny. If present, make smears and stain by Gram's method and make subculture for further identification.

Differential Diagnosis.—1. Collect fluids in sterile containers or tubes.

2. Examine as soon as possible after collection.

3. Centrifuge at high speed (one hour in the case of clear fluids).

4. Prepare smears and cultures from sediment.

5. Stain smears with Gram's stain. Clear fluids should also be stained for acid-fast bacilli, planted on Corper's medium, and inoculated into guinea-pigs if tuberculosis is suspected.

6. Staining characteristics:

Gram-negative { Meningococcus
B. influenzae

Gram-positive { Pneumococcus
Streptococcus
Staphylococcus

Acid-fast: Tubercle bacilli

Examination for Carriers.—1. The material for examination should be collected from the nasopharynx, particularly the posterior part.

2. By means of a bent applicator or West tube, material is collected from nasopharynx. Avoid touching soft palate or other structure.

3. Immediately inoculate the material on blood or sheep serum dextrose agar and place in incubator at once. The plates should be warm when inoculated. This can be done by keeping them in the incubator ready for use.

4. Meningococcus-like cultures should be differentiated from other gram-negative cocci, particularly *M. catarrhalis, M. flavus* and *M pharyngis-siccus,* by sugar fermentation and agglutination tests:

Organism	Glucose	Maltose	Mannitose	Levulose	Saccharose
Meningococcus	+	+	−	−	−
M. catarrhalis	−	−	−	−	−
M. flavus .	+	+	+	+	+
M. pharyngis .	+	+	+	+	+

Macroscopic Agglutination Tests for Meningococci.—1. Prepare with 0.85 per cent salt solution, 1 : 10 dilutions of normal and antimeningococcus horse serum for this purpose, and place a loopful of each on a glass slide.

2. Emulsify some of the colony in the drop containing normal serum and then transfer some of this suspension to the one containing antimeningococcus horse serum.

3. Examine for clumping of the organisms in the antimeningococcus serum.

4. If the organisms are gram-negative and are agglutinated in the antimeningococcus serum only, consider them to be meningococci.

A *macroscopic tube test* is conducted as follows:

1. Make a heavy suspension of a density of the barium sulphate nephelometer standard No. 3, using the culture grown on blood agar slants, or serum-dextrose agar slants, in o 85 per cent salt solution.

2. Make a 1 : 25 dilution of normal horse serum, and dilutions of the polyvalent antimeningococcus horse serum and monovalent antimeningococcus rabbit sera from animals immunized against groups I, II and III, according to the titer of the serum.

3. Put 0.3 c.c. of each of the serum dilutions in agglutination tubes and add 0.3 c.c. of the suspension of the organisms.

4. As an additional control, use 0.3 c.c. of the culture suspension and 0.3 c.c. of 0.85 per cent salt solution.

5. Shake the tubes thoroughly and incubate for twenty-four hours at 55° C.

6. If the organisms tested are meningococci, they should be strongly agglutinated by the polyvalent horse serum, and by the monovalent rabbit serum of the homologous type. They should not be agglutinated in the salt solution, and should be only very slightly agglutinated in normal horse serum, diluted 1 : 50.

7. There are nonpathogenic gram-negative cocci found in the nasopharynx that are strongly agglutinated by the normal horse serum, diluted 1 : 50.

BACTERIOLOGICAL DIAGNOSIS OF M. CATARRHALIS (NEISSERIA CATARRHALIS) INFECTIONS

Smears.—1. Prepare smears from mucous membrane.

2. Fix and stain by Gram's method.

The *M. catarrhalis* appears in pairs and sometimes fours. They are gram-negative; coffee bean in shape, resembling gonococci but slightly larger; non-motile.

Cultures.—1. Spread or streak exudate from mucous membrane on blood agar plates. Slants may be used if plates are not available or the patient is some distance from laboratory. The organism will grow on plain agar but not so well as on blood agar.

2. Incubate plates for twenty-four hours.

3. The colonies are small, gray or yellowish-white, circular with irregular

borders; they have a mortar-like consistency. Gelatin is not liquefied. Milk is not coagulated. Make smears and subcultures from suspicious colonies.

For further differentiation from other gram-negative cocci by sugar fermentation tests, especially the meningococcus, see page 348.

These organisms may be found in the secretions of normal mucous membranes and in certain diseased conditions may be abundant, especially in subacute and chronic infections of the nose, nasopharynx and bronchi.

IDENTIFICATION OF SPORE-BEARING AEROBIC GRAM-POSITIVE BACILLI

Anthrax group: *B. anthracis*
Mycoides group: *B. mycoides; B. ramosus*
Subtilis group: Hay bacillus (*B. subtilis*)

	Anthrax Group	Mycoides Group	Subtilis Group
Gelatin stab	Branching along stab	Branching along stab	No branching
Bouillon	No membrane, non-motile	Thick membrane, slight motility	Membrane actively motile
Virulence..........	Virulent	Nonvirulent	Nonvirulent

B. anthracis is the only one of this group which is pathogenic for man, and can always be differentiated by demonstrating its pathogenicity by subcutaneous inoculation of guinea-pigs.

BACTERIOLOGICAL DIAGNOSIS OF ANTHRAX (B. ANTHRACIS)

Examination of Pustule.—1. Inoculate plain agar tubes, or better, plates, with material from the pustule. When taking material from a pustule before excision, avoid rough manipulation which might cause the bacteria to be expressed into the blood circulation. Exercise great care against contracting infection.

2. Make smears and stain by Gram's method. The anthrax bacillus is gram-positive when not decolorized too long; forms short chains, ends are square and may be encapsulated (Fig. 210). A diagnosis may be made from the smear alone, but this should be confirmed by culture.

CULTURE.—1. Inoculate plain agar.

2. Incubate twenty-four hours. The growth is rapid. The colonies are grayish white, flat and spreading, the edges showing filamentous projections. Under the low power they appear like waves of hair.

Blood Cultures.—The organisms may appear in the blood, especially in severe cases just before death. Blood cultures have a definite value in prognosis and when positive indicate active therapeutic measures. Human beings may die as a result of bacteriemia.

If blood cultures are made, at least 15 to 20 c.c. should be collected and cultured in plain broth.

Animal Inoculation.—1. Inoculate a guinea-pig or mouse with suspected material subcutaneously.

2. If anthrax bacilli are present, the animal will die in from one to four days.

3. Make smears of blood and organs and look for gram-positive bacilli in chains and with square ends.

4. Inoculate cultures for further identification.

5. The lesions produced are: a serogelatinous edema over the region of inoculation; a swollen and dark spleen, and the heart's blood is often very dark and tarry in appearance.
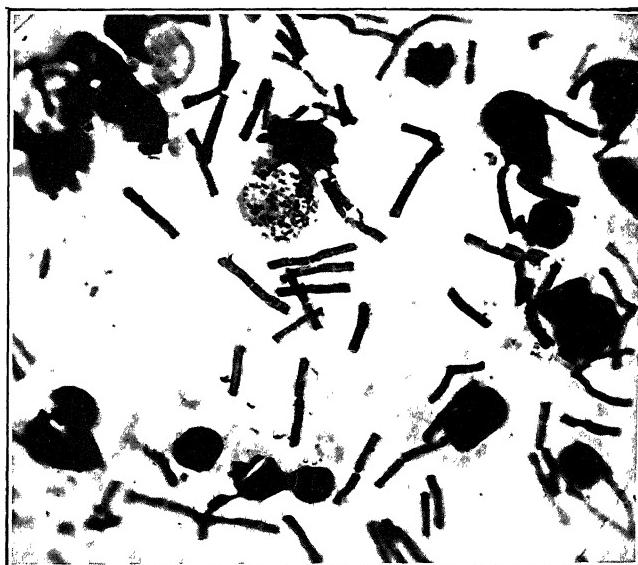
FIG. 210.—ANTHRAX BACILLI.
Smear of spleen of animal dead of anthrax. (Zinsser)

Differential Diagnosis.—*B. anthracis* may be mistaken for *B. subtilis* and *B. oedematis-maligni.* *B. subtilis* is actively motile and nonpathogenic; *B. anthracis* is nonmotile and virulent for mice, guinea-pigs and rabbits. *B. oedematis-maligni,* while motile, is a strict anaerobe and Gram-amphophil (usually positive).

Isolation from Hair or Bristles.—1. Rub up the suspected material in saline solution.

2. Heat one half at 80° C. for thirty minutes to kill non-spore-forming contaminants.

3. Centrifuge both portions.

4. Prepare four agar plates of both sediments.

5. Inoculate mice and guinea-pigs with both.

6. Study colonies (special attention to deep ones).

Precipitin Test (Ascoli).—1. This test is of special value in inspection of meat for anthrax.

2. Macerate the tissue and boil with 5 to 10 parts of saline solution or 1:1000 acetic acid in saline for fifteen minutes.

3. Centrifuge and filter through paper.

4. Place 0.5 c.c. of anti-anthrax serum of good precipitating titer in a small test tube and carefully overlay with 0.5 c.c. of clear extract.

5. A ring at the point of contact indicates a positive reaction.

6. When a good serum of known specificity is employed, the reaction is reliable

IDENTIFICATION OF THE SPORE-BEARING ANAEROBIC BACILLI
(STITT)

1. Rods very little swollen by centrally situated spores:
    (a) Motile: *B. sporogenes*
    (b) Nonmotile: *B. welchii* (capsule)
2. Spores tend to be situated between center and end:
    (a) Tends to form chains: *Vibrion septique*
    (b) Single or in pairs: *B. botulinus*
3. Spores situated at end of rod:
    Drumstick sporulation: *B tetani*

A study of the pathogenic effects of the organisms of this group is of considerable assistance in their identification. The following from Lehman and Neumann gives an outline of the effects produced by subcutaneous inoculation:

1. No particular symptoms at site of inoculation; absorption of the soluble toxin causing:
    (a) General symptoms of tetanus: *B. tetani*
    (b) Botulism-poisoning symptoms; pupillary symptoms; paralysis of tongue and pharynx. Cardiac and respiratory failure: *B. botulinus*
2. Local symptoms marked at site of inoculation. Hemorrhagic emphysematous edema:
    (a) Motile
        (1) Digests blood serum: *B sporogenes*
        (2) No action on blood serum: *Vibrion septique*
    (b) Nonmotile
        B. welchii

Animal Protection Test for Diagnosis of Gaseous and Phlegmonous Gangrene.—1. Prepare a saline extract of macerated gangrenous tissue or wound secretion.

2. Place 1 c.c. into each of five small sterile test tubes.

3. Add 1 c.c. of the following antisera:

    No. 1: Tetanus antitoxin

    No. 2: Anti-welchii serum (*B. welchii*)

    No. 3: Anti-malignant edema serum (*Vibrion septique*)

    No. 4: Anti-oedematiens serum (*B. oedematiens*)

    No. 5: Saline solution (control)

4. Place in incubator for thirty minutes.

5. Inject into five guinea-pigs (subcutaneously) respectively.

6. The control becomes sick in six to twelve hours and usually dies.

7. The one or ones protected by serum show no reactions and indicate the nature of the infection.

BACTERIOLOGICAL DIAGNOSIS OF TETANUS (CLOSTRIDIUM TETANI)

Smears.—1. Make smears from each wound or points of suppuration.

2. Stain with carbolfuchsin.
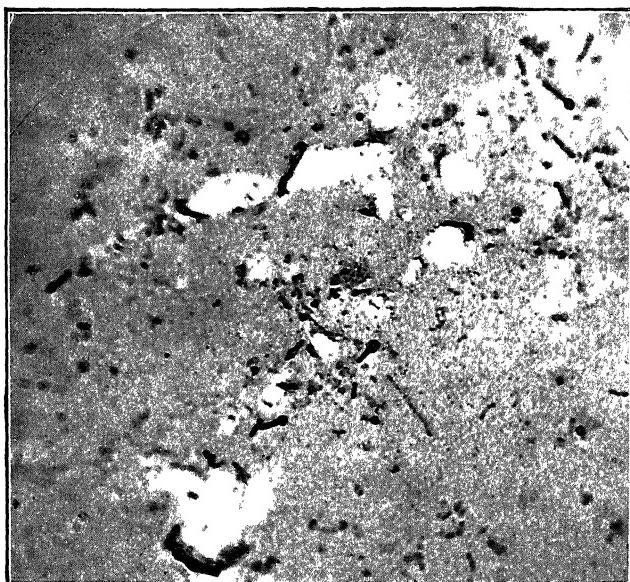
Fig 211.—Tetanus Bacilli; Spore Stain (Zinsser.)

3. Look for the typical spore-bearing or drumstick forms (Fig. 211). These are rarely found by direct smear. Note the presence of other bacteria.

Culture.—1. Pieces of tissue, pus, etc., are dropped into glucose bouillon, to which is added a small piece of fresh tissue. See method for culturing anaerobic organisms, page 303.

2. If material is probably contaminated with tetanus spores and other organisms, prepare an emulsion in broth, heat to 80° C. for thirty minutes in a water bath (kills all except spore-bearing bacilli), and culture.

Animal Inoculation.—1. Inoculate a mouse or guinea-pig subcutaneously with saline suspensions of pus, bits of tissue, etc., from wound.

2. If tetanus bacilli or spores are present in the material inoculated, the animal will develop, in from one to four days, symptoms of tetanus. In mice and guinea-pigs the muscles nearest the point of injection are first affected. At autopsy, no changes are found except a hemorrhagic spot at site of injection or suppuration due to other bacilli present in the inoculum.

BACTERIOLOGICAL DIAGNOSIS OF MALIGNANT EDEMA (CLOSTRIDIUM OEDEMATIS-MALIGNI)

Smear.—Prepare smears of the frothy, sour serous fluid and stain by Gram's method. *B oedematis-maligni* (*Vibrion septique*) is Gram-amphophil; may show very narrow and faint capsule; occurs singly or in short chains. Wet preparations show motile bacilli.

Culture.—Boil a tube of glucose agar and cool to 40° C. Inoculate with exudate and cover with sterile vaselin, paraffin oil, or molten agar (the bacillus is an obligate anaerobe). Incubate twenty-four to forty-eight hours.

Gas and acid are produced. Long bacilli in smears are found end to end in pairs and chains. Spores may be found. No capsules; motility present.

Animal Inoculation.—Cultures or exudates are highly pathogenic for young guinea-pigs, mice and rabbits.

BACTERIOLOGICAL DIAGNOSIS OF GAS WOUND INFECTIONS (CLOSTRIDIUM WELCHII)

Culture.—1. With sterile applicator obtain material from wound. Use a separate applicator for each part of wound examined. (Always examine several parts, especially the deeper parts.)

2. Place applicator in sterile test tube for purpose of carrying or storing until media are inoculated.

3. Boil glucose agar tubes for ten minutes and cool quickly to 42° C.

4. Inoculate agar by rubbing swab in it. See that material on swab is well distributed through the agar.

5. Solidify agar in ice box.

6. Incubate at 37° C.

7. If gas bacilli (*B welchii*) are present, gas will form rapidly after twelve to eighteen hours. The tube will be filled with gas bubbles and broken up.

8. Make smears from culture and stain by Gram's method. Look for

rather large gram-positive encapsulated bacilli (Fig. 212). For differentiating the gram-positive anaerobes, see page 351.

Animal Inoculation.—1. Inject a rabbit intravenously with 1 to 3 c c of saline emulsion of wound secretion or broth culture.

2. Kill animal five minutes later and place in incubator for sixteen hours.

3. Prepare smears of heart blood and liver. Stain by Gram's method.

4. Prepare cultures of heart blood and liver in milk.

5. If "stormy fermentation" occurs after incubation of twenty-four hours,
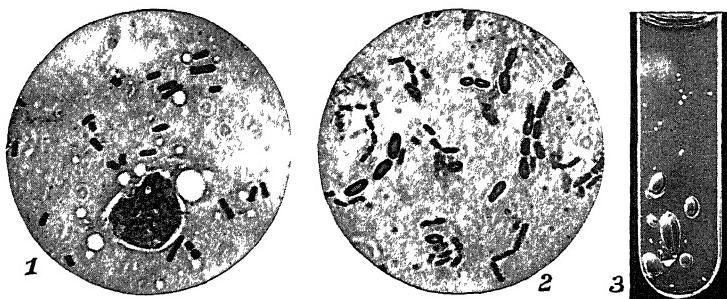
FIG 212—CLOSTRIDIUM WELCHII.

1, Smear from wound   2, Smear from culture.   3, Culture tube showing gas formation in agar.

(From Park, Williams and Krumwiede, *Pathogenic Microorganisms,* Lea and Febiger)

the presence of *B. welchii* may be assumed. Prepare anaerobic plate cultures for isolation.

BACTERIOLOGICAL DIAGNOSIS OF DIPHTHERIA
(CORYNEBACTERIUM DIPHTHERIAE)

Smears.—1. By means of a cotton swab or platinum loop secure a small bit of exudate or membrane to be examined.

2. Make thin smears on slides.

3. Stain with Loffler's methylene blue for one or two minutes. Neisser's stain is also recommended for demonstrating granules (see page 313).

4. Wash with water, dry, and examine with oil-immersion objective.

5. The diphtheria bacilli will appear as rather short, irregular staining, occasionally club-shaped bacilli arranged parallel or around a common center like the spokes of a wheel. They can be divided into three main types—solid, barred, and granular.

6. If diphtheria bacilli are not found, stain a slide for Vincent's angina infection.

7. The absence of diphtheria bacilli does not exclude the possibility of the infection, as bacilli may appear in culture.

Cultures.—1. Loffler's blood serum is the medium of choice.

2. Incubate at 37° C. for eighteen to twenty-four hours. In eighteen to twenty-four hours the diphtheria bacilli produce a luxuriant, yellowish-white or creamy growth, usually in the form of isolated colonies.

3. From this make a smear on a glass slide or cover glass and stain as described above. Be sure to pass the loop lightly over the entire surface of the slant in order not to miss isolated colonies.

4. The smear will often show a pure culture, owing to the fact that in the first eighteen to twenty-four hours the diphtheria bacilli outgrow the other
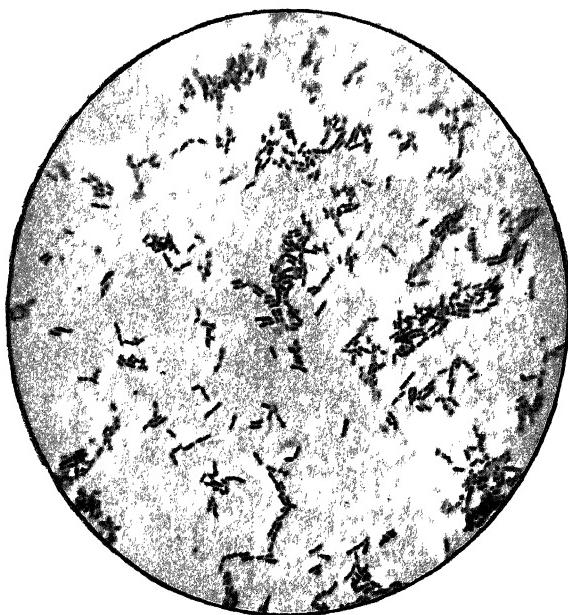
FIG 213—DIPHTHERIA BACILLI (Wood)

organisms (Fig. 213). For differentiation from diphtheroids, see page 358. If in doubt, stain smears by the Neisser and Gram methods.

Subcutaneous Virulence Tests.—1. If the culture on a slant of Loffler's medium appears to be pure, wash it off with 10 c c. of sterile saline solution, emulsify and inject a 250 to 300 gram guinea-pig (not heavier) subcutaneously with 4 c c in the median abdominal line. Inject a second pig with the same amount plus 1 c.c. of antitoxin (100 to 500 units).

2. If the culture is not pure, first isolate the diphtheria bacilli by the "streak" method on plates of Loffler's blood serum, ascitic or blood agar.

3 Inoculate a tube of glucose broth with several different colonies. If one colony is used, there is danger of picking up a nonvirulent organism and

securing a negative result even though virulent bacilli are present in the original culture.

4. Incubate at 37° C. for forty-eight hours, keeping the tube in a slanted position to give the culture as much oxygen as possible.

5. Examine for purity. Inject a 250 to 300 gram pig subcutaneously in the median abdominal line with 2 c c. of culture. Inject a second pig with the same amount plus 1 c.c. of antitoxin (100 to 500 units).

6. Observe for at least four days. The control animal by either method should survive and show no edema at the site of inoculation.

7. Virulent bacilli by either method will kill the pigs within four days with marked hyperemia of the suprarenal glands. Or the pigs will be sick and show marked edema at the site of inoculation; this is also a positive result even though the pigs may not succumb in four days (they are likely to develop paralysis of the hind legs later on).

Intracutaneous Virulence Tests.—1. The advantage of this method is that two or three cultures can be tested with one guinea-pig or rabbit. It is, however, less reliable than the subcutaneous tests.

2. Denude an area of abdominal skin by plucking out the hair for injection of each culture.

3. Emulsify a twenty-four-hour growth on a Loffler slant in 10 c.c. of sterile saline solution.

4. Inject 0.15 c.c. intracutaneously.

5. Virulent bacilli produce a positive reaction of definite local inflammation in twenty-four hours which goes on to superficial necrosis in forty-eight to seventy-two hours.

6. A control animal may be inoculated in the same manner, but receiving an intraperitoneal injection of 100 to 500 units of antitoxin. It should show no lesions.

BACTERIOLOGICAL DIAGNOSIS OF B. XEROSIS (CORYNEBACTERIUM XEROSIS) INFECTION

1. This is the special name given to the diphtheroid bacillus of the eye.

2. The bacillus may be found on the normal conjunctivae; it sometimes produces a low-grade chronic conjunctivitis.

3. Smears of conjunctival secretion stained by Löffler's methylene blue or Gram's method show solid bacilli of typical grouping and morphology.

4. Cultures on Loffler's blood serum or blood agar show luxuriant growths of large, solid, gram-positive bacilli of typical grouping.

5. Cultures are nonvirulent for guinea-pigs.

6. Cultures usually ferment some sugars in Hiss's serum water medium, especially saccharose.

DIFFERENTIATION OF DIPHTHERIA BACILLI FROM HOFFMAN'S BACILLUS (CORYNEBACTERIUM HOFFMANNI) AND B. XEROSIS

1. *B. hoffmanni* is the special name given a nonvirulent diphtheria-like bacillus to be found in normal throats and noses (Fig. 214).

FIG. 214—BACILLUS HOFFMANNI (Wood)

2. Nonvirulent or pseudodiphtheria bacilli (diphtheroids) may also occur in the pus of chronic infections (like otitis media and chronic prostatitis), on the skin, in lymph glands, and elsewhere

3. These organisms are usually shorter, thicker, and more uniform in size than the diphtheria bacillus They stain solid or with an unstained band across the middle and show no granules.

4. They grow more luxuriously than diphtheria bacillus on plain and blood agar although usually less so in broth.

5. They are differentiated from the diphtheria bacillus by the sugar fermentation and virulence tests and to some extent by morphology and more luxuriant growth on plain agar. The Hiss serum water medium with the following sugars is recommended:

Organism	Virulence	Dextrose	Dextrin	Saccharose
B diphtheriae (virulent)	+	+	+	−
B. diphtheriae (nonvirulent).	−	+	+	−
B. hoffmanni (diphtheroid)	−	−	−	−
B. xerosis	−	+	−	+

BACTERIOLOGICAL DIAGNOSIS OF VINCENT'S ANGINA (BORRELIA VINCENTI AND FUSIFORMIS DENTIUM)

1. Make smears on glass slides (not too thin).
2. Dry in air.
3. Fix by passing through flame four times.
4. Cover with carbolfuchsin diluted 1 : 10 with water; heat gently and stain for two minutes. Stain second slide by method of Gram.
5. Wash in water and dry.
6. Examine with oil-immersion lens for fusiform bacilli and spirilla. The former are gram-negative, long, slightly curved with pointed ends and showing faintly staining granules. The latter are large, wavy spirals (Fig. 215).

FIG. 215.—FUSIFORM BACILLI AND SPIRILLA OF VINCENT'S ANGINA (Zinsser.)

7. Both organisms are also readily seen in wet preparations examined with high dry or oil-immersion objectives.

8. Both organisms are strict anaerobes; cultures are not employed for diagnosis.

BACTERIOLOGICAL DIAGNOSIS OF TUBERCULOSIS
(MYCOBACTERIUM TUBERCULOSIS)

Detection of Tubercle Bacilli in Sputum by Smear Examination.—1. Pour the sputum into a Petri dish and pick up with sterilized platinum wire small white or yellow caseous particles; if none are present, choose for examination some of the thicker yellowish or greenish portions.

2. Make at least two smears on glass slides. They should be thin and uniform; never heavy and unevenly distributed. Material may be put on the upper half of a slide and squeezed out with another slide, continuing the rubbing until the sputum is evenly distributed when the slides are separated.

3. Stain with Ziehl-Neelsen's carbolfuchsin for acid-fast organisms, as described on page 309.

4. The tubercle bacilli will appear as red, solid or vacuolated, straight or slightly curved rods (see Plate IV); other bacteria and cells are stained blue At least two smears should be examined before a negative report is given and five minutes or more devoted to the examination of each.

SOURCES OF ERROR.—1. Scratches in the slides may retain the stain and be mistaken for acid-fast bacilli.

2. Incomplete decolorization.

3. There may be tubercle bacilli in the carbolfuchsin washed off from former specimens if the stain is being repeatedly used.

4. Acid-fast bacilli may be present in stale distilled water used for washing slides; also in vaselin and milk bottles used for collection of specimens.

5. Wood fibers, food particles and crystals may retain the fuchsin and re-

semble tubercle bacilli, although the latter are usually readily differentiated by careful study of morphology.

Detection of Tubercle Bacilli in Urine by Smear Examination.—1. Collect the specimen as described on page 258.

2. Shake the specimen of urine thoroughly, fill two centrifuge tubes, 50 c.c. capacity, and centrifuge (if smaller tubes are used more will have to be used or the centrifuging repeated until sediment from at least 100 c.c. of the urine has been collected).

3. Mix the sediment from both tubes or the sediment from 100 c.c. of urine. (If sediment contains crystals, they should be dissolved by very small amounts of either ammonium hydroxide if acid, or acetic acid if alkaline, diluted with water and centrifuged.)

4. By means of platinum loop, transfer sediment to slides.

5. Spread out in thin films and dry.

6. Fix by gentle heat.

7. Stain for acid-fast bacilli by Ziehl-Neelsen method.

8. It may be necessary to add a drop of Mayer's egg albumin to the sediment on the slide, before spreading, to facilitate adherence.

9. An important source of error is the presence of smegma bacilli.

Detection of Tubercle Bacilli in Feces by Smear Examination.—1. Make thin smear of feces on slide or cover glass; if blood or mucus is present in the feces, this will be more apt to contain bacilli.

2. Dry in air, fix, and stain for acid-fast bacilli, in the same manner as outlined for sputum examination.

3. The antiformin methods are a great aid and may be conducted in the same manner as for the examination of sputum.

4. Petroff recommends diluting the stool with 2 volumes of water, stirring and filtering to remove the coarse particles. The liquid stool is then saturated with sodium chloride crystals and allowed to stand at room temperature for several hours. Collect the scum with a sterile spoon and place in a wide-mouthed bottle. Add 2 volumes of normal sodium hydroxide, shake well and incubate at 38° C. for one to two hours. Centrifuge and decant the supernatant fluid. To the sediment add 3 to 4 drops of normal hydrochloric acid. One part may be smeared on tubes of gentian-violet-egg medium for culture, a part used for guinea-pig inoculation and a part for making smears to be stained by the usual methods.

Detection of Tubercle Bacilli in Blood by Smear Examination.—1. Draw 10 c.c. of blood from a vein and place in 100 c.c. of sterile distilled water.

2. Add a few drops of 5 per cent tannic acid solution.

3. Mix and place in the refrigerator for several hours.

4. Centrifuge and decant the supernatant fluid.

5. Prepare and stain smears of the sediment. Portions may be used for inoculation of guinea-pigs.

Detection of Tubercle Bacilli in Pleural and Spinal Fluids by Smear

Examination.—1. Collect and prepare smears as described above for urine; if a coagulum has formed, remove and tease out on a slide with pins.

2. Stain and examine for acid-fast bacilli. Prolonged search is generally required.

3. For differential diagnosis of spinal fluid, see page 238. Tubercle bacilli are the only acid-fast bacilli encountered in spinal fluids.

4. Petroff recommends the following method: To approximately 10 c.c. of fluid, add 2 drops of 5 per cent solution of tannic acid. Mix well and centrifuge. Decant the supernatant fluid. Prepare and stain smears of the sediment, portions of which may be also used for preparing cultures and inoculating guinea-pigs.

5. Pleural fluids containing large clots may be examined by separating the clot and adding to it equal volumes each of normal sodium hydroxide and 15 per cent solution of antiformin. Shake and digest at 37° C. for about an hour. Centrifuge and decant the supernatant fluid. To the sediment add a few drops of normal hydrochloric acid; prepare and stain smears. The sediment may also be used for cultures and guinea-pig inoculation.

Detection of Tubercle Bacilli in Milk.—1. Use milk as fresh as possible.

2. Centrifuge 30 c.c. at high speed.

3. Take off the cream on top, dilute 4 c.c. with sterile water and inoculate guinea-pigs subcutaneously.

4. Inject 1 to 3 c.c. of sediment into additional pigs. Also prepare and stain smears.

5. Acid-fast bacilli in smears may be "butter bacilli" and also produce local lesions but not generalized infections. To prevent these errors, inoculate tubes of glycerin agar with sediment or cream. Butter bacilli develop in a few days at 37° C. and also at room temperature.

6. When ready to examine the pigs, inoculate each with 2 c.c. of old tuberculin late in the day. The following morning the tuberculous animals will be dead or dying; conduct autopsies to confirm the results.

Detection of Tubercle Bacilli by Concentration Methods.—ANTIFORMIN METHOD.—This is considered useful when direct smears are negative. Examination of a sample of the entire twenty-four-hour sputum is recommended. Antiformin is a proprietary preparation and may be prepared by mixing equal parts of liquor sodae chlorinatae, U. S. P., and a 15 per cent solution of sodium hydroxide. The former is prepared by dissolving 600 grams of sodium carbonate and 400 grams of chlorinated lime in 4000 c.c. of distilled water, allowing the mixture to settle and then filtering the supernatant fluid. Equal parts are mixed with a 15 per cent solution of sodium hydroxide. Keep in a dark bottle in a cool place.

1. Place equal parts of sputum and 50 per cent antiformin solution in small beaker or prepare the mixture in the sputum container.

2. Incubate at 37° C. for thirty minutes, stirring occasionally to insure complete liquefaction or shake for thirty minutes in a shaking machine.

3. Dilute with 3 volumes of sterile water to reduce specific gravity of the solution.

4. Centrifuge for ten to thirty minutes; pour off supernatant fluid; fill tube with water; centrifuge; continue until all of the fluid has been centrifuged.

5. To sediment left, after pouring off supernatant fluid, add sterile distilled water, mix well, centrifuge and pour off supernatant fluid.

6. By means of a platinum loop transfer sediment to slides.

7. Make smears and stain for acid-fast bacilli.

8. If the sediment does not adhere to the slide, apply a thin smear of Mayer's albumin or raw egg albumin to the slide (egg white, 1 part; water, 10 parts, and formalin, 1 part) and spread the sediment.

9. The sediment may be used for cultural purposes or for inoculation of guinea-pigs, although some tubercle bacilli may be destroyed.

PETROFF'S METHOD.—Instead of using antiformin, Petroff recommends adding to the sputum an equal volume of 4 per cent sodium hydroxide. The mixture is kept at 38° C. for fifteen to thirty minutes, shaking frequently to insure a uniform mixture. After complete homogenization, it is centrifuged at high speed and the supernatant fluid decanted. To the sediment add 2 or 3 drops of normal hydrochloric acid to make it slightly acid and prepare smears as above.

Petroff's method is conducted with twenty-four-hour specimens. Acidulate with a few drops of 30 per cent nitric acid. To every 1000 c.c. of urine, add 2 c.c. of 5 per cent tannic acid solution. Shake well and place in refrigerator for twenty-four hours. Decant the supernatant fluid and centrifuge the sediment; decant the supernatant fluid. Treat the sediment with 1 c.c. of normal sodium hydroxide solution, which should completely dissolve it. Warm for thirty minutes at 37° C.; dilute with 3 volumes of sterile water and centrifuge. Decant, prepare and stain smears of the sediment. If cultures are to be made, add 5 volumes of normal sodium hydroxide to the sediment; shake well, incubate for thirty minutes and centrifuge at high speed for five to ten minutes. Decant the supernatant fluid, add 2 drops of normal hydrochloric acid, mix well and distribute the sediment on the surface of tubes of gentian-violet-egg medium. Paraffin the stoppers and incubate. Tubercle bacilli if present will appear in about two weeks.

LIGROINE METHOD.—1. Place equal parts of sputum and undiluted antiformin in a bottle and add 1 to 2 c.c. of ligroine with a specific gravity of 7.18 to 7.20.

2. Shake well for twenty minutes. (If the ligroine is below 7.16 now place container on ice for thirty minutes to aid separation.)

3. Centrifuge at moderate speed for ten minutes and prepare smears from the under side of the "blanket" which appears between the digested material and the ligroine.

· UHLENHUTH'S METHOD.—This is conducted by centrifuging the urine as described; treating the sediment with antiformin in the proportion of 1 to 4,

or 1 to 2 volumes and stirring until it is perfectly homogeneous. The specimen is then centrifuged for ten minutes; the supernatant fluid poured off and the sediment washed two or three times with saline solution, each time by stirring with a loop, followed by centrifuging and decanting. Smears are then made on slides (use Mayer's albumin solution for fixative if the urine contains no albumin and but few pus cells). Dry in the air and stain with carbolfuchsin by heating gently for ten minutes. Decolorize with acid alcohol and counterstain with methylene blue.

Cultural Method of Isolating Tubercle Bacilli.—The cultural method of Corper is recommended and conducted as follows:

1. Use Corper's glycerol water crystal-violet potato medium described in Chapter XVII.

2. One c.c. of suspected material is beaten to a homogeneous pulp and introduced into a sterile centrifuge tube of 15 c.c. capacity with 1 c.c. of 6 per cent sulphuric acid containing 17 c.c. of 96 per cent (specific gravity, 1.84) sulphuric acid in 500 c.c. of distilled water) or, better, 1 c.c. of 5 per cent pure oxalic acid (by weight). After incubation at 37° C. for thirty minutes, the contents of the tube are mixed with about 10 c.c. of sterile 0.9 per cent sodium chloride solution and centrifugalized. The residue, after the supernatant fluid has been decanted, is seeded lightly on the surface of three to six tubes of the medium, the culture tube being capped with tin-foil after the cotton plug has been lightly impregnated with hot paraffin to prevent drying out of the medium.

3. The culture tubes should be incubated in the dark, with due precaution being taken to avoid drying of the medium or contamination. A luxuriant growth should occur on this medium within from two to six weeks; but if the culture is negative, the tubes should not be discarded for diagnostic purposes until after three months' observation at incubator temperature.

4. A few of the ordinary precautions necessary in growing tubercle bacilli by the new culture method are: (*a*) Avoid changes which may occur in the potatoes before autoclaving by not allowing them to stand too long a time after cutting. (*b*) Avoid drying of the culture medium during the long period of incubation necessary by paraffining the cotton stoppers. (*c*) Keep the culture tubes while in the incubator in a dark receptacle like a covered tin can or keep the incubator dark, preferably both.

5. Tubercle bacilli in exudates or other material contaminated with other organisms may be isolated by injecting the material into a guinea-pig and then obtaining material aseptically from the tuberculous lesions. Such material will contain many more tubercle bacilli and none of the contaminating organisms. Otherwise the tubercle bacilli grow slowly and may be readily overgrown by contaminating organisms.

Detection of Tubercle Bacilli by Animal Inoculation.—1. Morning sputum is preferred and inoculated at once or after washing with sterile saline solution. If not fresh and if many other bacteria are present, it may be digested with an equal volume of 2 per cent sodium hydroxide and neutralized with

an equal volume of 5 per cent oxalic acid to avoid septic infection of the animals. If urine is to be examined, collect sediment from 100 c.c. Feces should be digested in the same manner as sputum.

2. Weigh two guinea-pigs and note their weights. Also note their color or otherwise mark them for further identification.

3. Inoculate each pig subcutaneously with the sediment from 50 c.c. of urine, a bean-sized portion of sputum or the washed sediment of feces after treatment with sodium hydroxide and oxalic acid as described above. Do not inject into the mammary glands.

4. Bloch recommends damaging the inguinal lymph glands by squeezing between the finger, and injection of the material into these damaged glands.

5. Examine the animals each week for symptoms of tuberculosis. Weigh them and examine site of inoculation for tubercles or tuberculous ulceration, also superficial lymph glands or enlargements. If the subcutaneous glands are enlarged, obtain pus and examine by smears for tubercle bacilli.

6. If the animals show physical signs of tuberculosis at the end of two weeks, one may be killed and the presence of tuberculosis confirmed, in which case the other animal may be destroyed and examined. If necropsy of first animal fails to reveal tuberculosis, allow the other to live for four to six weeks, then destroy, examine and report positive or negative findings. Or at the end of two weeks inject one of the animals intracutaneously with 0.1 c.c. of a 5 per cent solution of old tuberculin. If no reaction results in forty-eight hours, repeat the test two weeks later.

7. Should neither pig show any signs of tuberculosis, one should be allowed to live four to six weeks before destroying and the other for a longer period up to at least three months. It sometimes happens that specimens of urine contain organisms other than tubercle bacilli in large numbers which cause the death of the inoculated animals in a few days and require a repetition of the test.

8. Enlarged glands or tissue from other organs (spleen) may be examined for tubercle bacilli by making smears and staining. If acid-fast bacilli are not found, the tissues should be examined histologically for tuberculosis before a negative report is warranted. Smears made from the site of inoculation are especially important.

9. Never base a negative report alone on the absence of enlarged external glands; the internal glands and spleen require examination.

10. The two outstanding disadvantages to the use of the guinea-pig as compared to the culture method are: (a) The guinea-pig is an indirect diagnostic criterion, revealing only tuberculous disease which as such may at times be confused with other spontaneous infections in these animals. (b) The guinea-pig possesses the decided possibility of developing tuberculous disease from extraneous sources, vitiating its definite value considerably. In addition the culture method possesses the advantages of economy, makes immediately accessible a culture for differentiating human from bovine bacilli, should this be desired, while a culture must first be obtained for this purpose if the guinea-

pig is used as test, and if generalized disease in the guinea-pig is used as the criterion of a positive diagnosis, the culture method, revealing tubercle bacilli, becomes positive several weeks earlier.

BACTERIOLOGICAL DIAGNOSIS OF LEPROSY (MYCOBACTERIUM LEPRAE)

Examination of Skin Lesions by Smears.—1. Scrape a nodule or spot deeply with scalpel until epidermis is removed and serum exudes.

2. Prepare smears of the serum and stain by Ziehl-Neelsen's method for acid-fast bacilli. Twenty per cent sulphuric acid is preferred by some instead of acid alcohol for decolorizing. The lepra bacilli are less resistant to acid alcohol than the tubercle bacillus. Be careful not to carry decolorization too far.

3. Lepra bacilli are small, slender, acid-fast rods resembling tubercle bacilli in form, but somewhat shorter and not so frequently curved. Many of the bacilli are seen arranged in masses in the so-called "lepra cells." There are no staining or morphological differences between them and tubercle bacilli which permit positive differentiation. But large numbers of acid-fast bacilli, especially if occurring in cells, found in material scraped from tissues showing clinical evidences of leprosy, are for practical purposes lepra bacilli. The absence of acid-fast bacilli in scrapings or sections of tissue indicates very strongly, but does not definitely prove, that the material is not leprous. The bacilli are found especially in the nodular type and in ulcerative lesions. There may be few or no bacilli found in material from the anesthetic erythematous lesions.

4. Smears of the nose and throat stained as above may also show lepra bacilli. If coryza does not exist, the patient may be given 60 grains of potassium iodide to produce a discharge from which smears are prepared.

5. Sections of leprous nodules not only show the typical histological changes of the disease, but also large numbers of acid-fast lepra bacilli mostly in "lepra cells."

DIFFERENTIATION OF GRAM-NEGATIVE AEROBIC BACILLI THAT ARE DIFFICULT TO CULTURE (HEMOPHILIC BACTERIA)

All the organisms belonging to this group require either blood, serum, or both in the media. They produce minute dewdrop colonies. The following key is taken from Stitt's *Practical Bacteriology, Blood Work and Animal Parasitology:*

Do Not Grow on Ordinary Media.—Require blood agar (hemophilic bacteria), serum agar, or blood serum. Minute dewdrop colonies:

1. Influenza bacillus (Pfeiffer). Requires blood media.

2. Koch-Weeks bacillus (conjunctivitis). Serum agar best medium. Many, however, regard hemoglobin as necessary for growth.

3. Morax-Axenfeld diplobacillus of conjunctivitis. Grows well and produces little pits of liquefaction on Loffler's blood serum.

4. Bordet-Gengou bacillus of whooping cough Does not grow on Loffler's serum. Requires blood or ascitic fluid agar. Original isolation should be on glycerol-potato agar

5. Ducrey's bacillus (soft chancre). Requires media rich in blood or serum. Forms chains

6. *Bacterium tularense.* Grows only on coagulated egg yolk, cystin agar or blood agar containing sterile animal tissue.
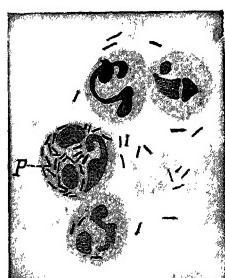
FIG 216 — CONJUNC-
TIVAL SMEAR SHOW-
ING KOCH - WEEKS
BACILLI

P, intracellular ba-
cilli.

(From Todd and Sanford,
*Clinical Diagnosis by Lab-
oratory Methods,* W B
Saunders Co)

BACTERIOLOGICAL DIAGNOSIS OF ACUTE CONTAGIOUS CONJUNCTIVITIS (PINK-EYE)

Smears.—1. Prepare smears from conjunctival secretion and stain by Gram's method

2. Examine for Koch-Weeks bacillus (*Hemophilus conjunctivitidis*) which is a small gram-negative bacillus slightly larger than *B influenzae*. Usually intracellular but also extracellular (Fig 216). In acute cases these organisms are present in large numbers; they tend to occur in shoals near the pus cells.

Cultures.—1. Inoculate blood agar slants if plates are not available, or, better, a mixture of glycerin agar and ascitic fluid with the exudate collected from bottom of conjunctival sac.

2. After forty-eight hours' incubation examine the cultures for very small dewdrop-like colonies of gram-negative bacilli.

BACTERIOLOGICAL DIAGNOSIS OF MORAX-AXENFELD (HEMOPHILUS LUCUNATUS) CONJUNCTIVITIS (ANGULAR CONJUNCTIVITIS)

1. Prepare smears and cultures on blood agar or Löffler's serum.

2. Stain slides by Gram's method

FIG 217.—MORAX-AXENFELD DIPLOBACILLUS
(Zinsser)

and look for gram-negative bacilli, which are a little longer but much thicker than the Koch-Weeks bacillus. They usually occur in pairs or short chains (Fig. 217).

3. Examine the cultures after twenty-four hours for gram-negative bacilli, which when grown on Löffler's blood serum, produce little pits.

BACTERIOLOGICAL DIAGNOSIS OF INFLUENZA

The following methods are recommended for the detection of B influenzae (*Hemophilus influenzae*), although the etiological significance of the organism is unsettled:

Smears.—1. Prepare smears from the posterior nasopharynx and fresh sputum. In cases of meningitis, centrifuge the cerebrospinal fluid and prepare smears of the sediment.

2. Stain by Gram's method.

3. Examine for small gram-negative bacilli, which stain rather lightly and occasionally show bipolar staining. They may occur in clusters and vary in form from coccoid to bacillary. Occasionally filamentous forms are seen, especially in spinal fluid.

Cultures.—1. Sputum should be obtained from the lower air passages and washed in sterile water. Select one or more solid particles from the sputum and spread on plates of either cholocate agar or Avery's sodium oleate agar. If other agar is used, a drop of fresh sterile human or rabbit blood should be placed on it and spread over the surface (blood—drop-agar plates). B. influenzae requires blood for its development. It is advisable to culture the nasopharynx as well as the sputum.

2. After twenty-four to forty-eight hours' incubation, examine for very small dewdrop-like colonies, which are colorless, transparent, and discrete. Suspicious colonies should be transplanted on chocolate agar and plain agar and examined for gram-negative bacilli.

FIG 218—BACILLUS INFLUENZAE. (Zinsser)

3. Examine the subcultures for growth after eighteen to twenty-four hours' incubation. A growth on the chocolate agar and no growth on the plain agar is characteristic of B influenzae.

4. The identification will depend upon the morphology (Fig. 218), staining reaction, appearance of colonies, and failure to grow in media lacking blood.

BACTERIOLOGICAL DIAGNOSIS OF WHOOPING COUGH (BORDET-GENGOU BACILLUS, HEMOPHILUS PERTUSSIS)

1. Examine sputum during onset of disease, as the Bordet-Gengou bacillus, considered as the cause of whooping cough, is usually to be found during the early stages.

2. When sputum is obtainable the more tenacious thick mucus should be chosen, taken up on a platinum loop, washed thoroughly in three baths of

sterile saline solution and plated by making light, even smears over an entire plate. With infants and young children from whom sputum may not be obtainable, an open plate may be held 5 or 6 inches in front of the mouth during a paroxysm. The former is preferred. Cultures of the nose and throat are of no value. Swabs of mucus gathered during coughing may be employed.

3. The best medium is the glycerin-potato-blood agar of Bordet-Gengou with a P^H of about 5.0. As this medium inhibits the growth of influenza bacilli, the isolation of the pertussis bacillus is facilitated. After isolation the culture can be maintained on unadjusted medium which has a P^H of 6.1 to 5.8.

4. Incubate at 35° C. to 37° C. for forty-eight hours. *B. influenzae* and other gram-negative bacilli closely resembling *B. pertussis* may be differentiated by complement-fixation tests and the following cultural characteristics (from Park, Williams and Krumwiede):

	Growth on Bordet-Gengou Plates	Bordet-Gengou Slants	Coagulated Horse-Blood Slants	Glycerin-Ascitic-Agar Slants	Plain Agar Slants
B. pertussis ...	Lightens the medium	First generation: in pure culture abundant non-spreading moist growth in twenty-four hours	After several generations: abundant tenacious growth in forty-eight hours	After several generations: abundant tenacious growth in forty-eight hours	After several generations: tenacious growth occurs slowly
B. influenzae...	Darkens the medium. No growth on acid Bordet-Gengou	First generation. delicate growth	First generation: abundant moist spread in twenty-four hours	Never grows	Never grows
Intermediate group of gram-negative bacilli	Markedly lightens the medium	First generation: abundant moist spread in twenty-four hours	First generation: profuse moist spread in twenty-four hours	First generation: profuse moist spread in twenty-four hours	First generation: profuse moist spread in twenty-four hours

5. Prepare smears and stain by Gram's method. Look for small, oval, gram-negative bipolar bacilli resembling somewhat *B. influenzae*.

Cough Plate Method.—1. Petri dishes carrying 20 c.c. of the same medium should be used. The blood should be added to the agar at about 45° C. to avoid dark or "chocolate" agar.

2. Expose an uncovered plate held 3 or 4 inches from the mouth at the moment of several *deep, expulsive coughs*. Two important causes of unsatisfactory results are that the plates are not well enough coughed on and that they are exposed too late in the disease.

3. Incubate the plates in an inverted position within a few hours after exposure.

4. Examine for colonies of *B. pertussis* at the end of the second day. The colony is translucent, markedly raised and definitely circular; size about 1 millimeter in diameter. Colonies may be surrounded by a characteristic darkened zone. They may not be found until the fourth or fifth days. Prepare smears and stain by Gram's method. *B. pertussis* appear as small, delicately staining, ovoid bacilli some of which show bipolar staining.

BACTERIOLOGICAL DIAGNOSIS OF TULAREMIA

1. Prepare smears and stain by method of Gram. *B. tularense* (*Pasteurella tularensis*) is gram-negative and is stained with difficulty.

2. Because of the difficulty with which the bacillus is cultivated, cultures are not recommended for obtaining the organism directly from the lesions for diagnosis. If attempted, however, use the blood-glucose-cystine agar (Francis) and incubate for at least three weeks. This medium is described in Chapter XVII for maintaining stock cultures for the agglutination test.

3. Inoculate a guinea-pig subcutaneously with material. Rub some of it on the skin of the abdomen of a second pig after abrading by shaving with a dull razor.

4. If the material contains *B. tularense,* the animals will usually die in from five to ten days. The lymph glands will be enlarged and caseous. Numerous white, caseous nodules will be found in the liver and spleen.

5. For diagnosis the agglutination test is recommended (see Chapter XXV for technic).

BACTERIOLOGICAL DIAGNOSIS OF CHANCROIDAL INFECTION (DUCREY'S BACILLUS, HEMOPHILUS OF DUCREY)

Smears.—Smears made from the lesion, particularly before ulceration sets in, may be of diagnostic value. These should be stained with carbolfuchsin or Gram's stain. The organisms stain best with the fuchsin, show polar staining, are small, gram-negative, and grow in chains.

Cultures.—1. Very rich blood media must be employed as the bacillus is difficult to cultivate and dies out very rapidly. The following have been recommended: (*a*) sterile clotted rabbit blood heated at 55° C. for fifteen minutes; (*b*) 2 parts of liquefied agar cooked to 50° C. with one part of sterile human, dog, or rabbit blood. Ordinary media are unsuitable.

2. Inoculate with pus from a dressing or aspirated from the margins of ulcers or buboes.

3. Incubate twenty-four to forty-eight hours and look for separated, well-developed, shining, grayish colonies with slight hemolysis and showing short, bipolar, nonmotile bacilli in chains and sometimes twisted in dense masses.

IDENTIFICATION OF GRAM-NEGATIVE BACILLI THAT ARE EASY TO CULTURE

1. **Organisms.**—The organisms included here are as follows:

   B. coli group: *B. coli-communis* and *B. coli-communior*
   B. cloacae group: *B. cloacae*
   B. lactis-aerogenes group: *B. aerogenes*
   Dysentery group: *B. dysenteriae* of Shiga, Flexner, etc.
   Friedländer's group: *B. pneumoniae, B. capsulatus, B. rhinoscleromatis*

Typhoid group: *B. typhosus*
Gartner group: *B. enteritidis, B. paratyphosus*
Proteus group: *Proteus vulgaris*
Chromogenic group: *B. pyocyaneus, B. prodigiosus* (readily identified by
their property of producing pigments).

These are classified as follows and may be differentiated according to the
accompanying table:

Genus Escherichia
Genus Aerobacter
Genus Proteus
Genus Salmonella
Genus Eberthella
Genus Shigella
Genus Klebsiella
Genus Pseudomonas

Medium	Escherichia	Aerobacter	Proteus	Salmonella	Eberthella	Alcaligenes
Litmus milk..	Pink	Pink	No change	No change	No change	No change
Glucose......	Acid and gas	Acid and gas	Acid and gas	Acid and gas	Acid	No change
Acetyl-methyl-carbinol*...	Not formed	Formed	Not formed	Not formed	Not formed	Not formed
Sucrose	o	o	Acid and gas	No change	o	o

* Voges-Proskauer reaction, see page 323.

2. **On Endo's or Conradi's Medium.**—Plates containing either of these
media are used for the isolation and differentiation of members of the colon,
typhoid, and intermediate groups of Gram-negative bacilli. The surface is
inoculated either by streaking or spreading.

The colon, cloacae and lactic acid-aerogenes groups produce red colonies.

The typhoid and other groups produce bluish or colorless colonies.

The suspected colonies are fished and subcultures subjected to further study.

3. **On Russel's Double Sugar Medium.**—This medium contains 1 per cent
lactose, 0.1 per cent glucose, and an indicator. It is useful for differentiating
members of the colon-typhoid-paratyphoid groups in pure culture. The me-
dium is inoculated by passing the loop over the surface of the slant and then
making a stab culture in the butt of the media. The principle is that bacilli
growing on the surface (aerobically) will only ferment sugar in the amounts
over 0.1 per cent, but when growing in the butt (anaerobically) they ferment
the small amount of glucose to obtain oxygen. The reaction is indicated by
the gas bubbles or change in the color of the media as follows:

Colon group: slant acid; butt acid and gas
Paratyphoid group: slant unchanged; butt acid and gas
Typhoid group: slant unchanged; butt acid only
B. alcaligenes: slant unchanged; butt unchanged

4. **Four Culture Method.**—Considerable information may be obtained for the identification of pure cultures by inoculating the following media: litmus milk, bouillon (for demonstrating motility), glucose medium (for demonstrating acid and gas), and gelatin. It should be remembered that many members of the various groups can only be definitely identified by more extensive study of cultural characteristics than given here or by serological tests:

	B. Coli Group	B. Cloacae Group	B. Lactis-Aerogenes Group	Dysentery Group
Litmus milk.............	Pink	Pink	Pink	No change
Motility........	Motile	Motile	Nonmotile	Nonmotile
Glucose............ ...	Acid and gas	Acid and gas	Acid and gas	Slightly acid
Gelatin..	No liquefaction	Liquefaction	No liquefaction	No liquefaction

	Friedländer's Group	Typhoid Group	Paratyphoid Group	Proteus Group
Litmus milk......	No change	No change	No change	No change
Motility..	Nonmotile	Motile	Motile	Motile
Glucose......	Acid and gas	Acid	Acid and gas	Acid and gas
Gelatin...	No liquefaction	No liquefaction	No liquefaction	Liquefaction

BACTERIOLOGICAL DIAGNOSIS OF B. COLI (ESCHERICHIA COLI) INFECTIONS

1. The material should be carefully collected in a sterile container; urine should be collected in a sterile container by catheterization.

2. Make smears and stain by Gram's method for gram-negative bacilli (Fig. 219).

3. Inoculate glucose broth with several c.c. of the material and incubate for one to two days.

4. Streak or spread the material or culture over surface of Endo plates and incubate for twenty-four hours.

5. If *B. coli* is present, the colonies will be red and show a metallic sheen. Pick several suspicious colonies and inoculate tubes of Russel's double sugar agar.

6. Tubes showing an acid slant and acid and gas in the butt can be further studied by inoculating litmus milk, glucose broth and testing for acetyl-methyl carbinol.

7. *B. coli* has been found as the cause of diarrhea, peritonitis, inflammation of the bile tract, pancreas, and urinary tract. It is a frequent cause of abscess, particularly in the region of the rectum, urethra, and kidney. Occasionally causes pneumonia, pleurisy, meningitis, conjunctivitis and endocarditis.

In lower animals it causes pyosepticemia (joint ill, navel ill) and dysenteria neonatorum (white scours) of sucklings.

FIG 219—BACILLUS COLI COMMUNIS. (Zinsser)

BACTERIOLOGICAL DIAGNOSIS OF B. PROTEUS (PROTEUS VULGARIS) INFECTIONS

1. Streak or spread material over surface of plain agar or blood agar plates.

2. The colonies of *B. proteus* spread very rapidly and often cover the entire plate with a very thin film. The bacillus usually overgrows other organisms that may be present and has a characteristic odor.

3. Subculture into litmus milk, glucose and saccharose media (see above table).

4. *B. proteus* is found in cases of cystitis, pyelonephritis, cholera infantum, otitis media and suppurative inflammations. It is commonly found in putrid meat and on account of its proteolytic power is a probable cause of food poisoning.

In health it is found on the skin, and often on the mucous membranes, where it may occur without doing any harm. On the nasal mucous membrane it decomposes the secretions and produces a foul odor.

Certain strains (*Proteus X19*) are agglutinated by the serum of patients suffering with typhus fever (see Chapter XXV).

BACTERIOLOGICAL EXAMINATION OF URINE FOR B. COLI (ESCHERICHIA COLI), B. TYPHOSUS (EBERTHELLA TYPHI) AND B. PARATYPHOSUS (SALMONELLA PARATYPHI AND SCHOTMUELLERI)

1. Collect urine in sterile container by catheterization under aseptic conditions.

2. Inoculate 8 to 10 c.c. of glucose broth with 2 to 5 c c. of urine.

3. Incubate for twenty-four to forty-eight hours.

4. Make smear and stain by Gram's method.

5. Examine for gram-negative bacilli; if present, plate on Endo's media or inoculate slant and butt of tube of Russel's double sugar medium.

6. Make subcultures and identify by cultural or serological methods (see four-tube method above) and agglutination tests on page 374.

7. If no growth occurs in forty-eight to seventy-two hours, specimen is sterile.

BACTERIOLOGICAL EXAMINATION OF FECES FOR TYPHOID AND PARATYPHOID BACILLI

1. Specimen should be collected in clean container, or better, a sterile one.

2. If specimen is solid, emulsify with a small amount of sterile water in a test tube. It should represent a generous amount of the whole specimen and not merely a loopful.

3. With a platinum loop streak three or four well-hardened plates of Endo's medium.

4. Incubate for twenty-four hours at 37° C.

5. Examine plates for suspicious colonies; colon group produces red colonies, typhoid and paratyphoid groups bluish-white or colorless colonies.

6. Fish five to ten suspicious colonies and make subcultures on Russel's double sugar agar.

7. Identification is accomplished serologically by agglutination tests of bouillon cultures with immune serum (see below) and bacteriologically by cultural and biological reactions (see four-tube culture method above).

BLOOD CULTURES FOR TYPHOID FEVER

Blood cultures are of particular value in the early diagnosis of typhoid during the first week or ten days.

1. Make blood cultures as described on page 259.

2. Inoculate glucose bouillon flasks containing about 200 c.c. of medium, with 5 to 10 c.c. of blood from patient.

3. Incubate for forty-eight to seventy-two hours.

4. Make smears and subcultures for identifying gram-negative bacilli (see four-tube method above).

AGGLUTINATION TESTS FOR THE IDENTIFICATION OF TYPHOID, PARATYPHOID, AND DYSENTERY BACILLI

Rapid Slide Methods.—These methods are recommended for the identification of typhoid bacilli in plates and their differentiation from colon bacilli; they are not suitable for differentiation between typhoid and paratyphoid bacilli. They may be likewise employed for the identification of dysentery bacilli, using monovalent or polyvalent immune sera.

1. For each colony or slant to be tested, arrange cover glasses and place on each a small drop of a suitable dilution of rabbit-typhoid-immune serum capable of giving strong agglutination with *B typhosus*. As a general rule this may be approximately 1 : 100.

2. Emulsify in a drop of diluted serum a small portion of the colony to be studied picked up with a platinum loop. Number the colonies to correspond to the slides.

3. At the same time prepare controls of each colony in the same manner, using saline solution instead of serum.

4. Suspend each preparation in hanging drop slides.

5. Examine microscopically in about fifteen minutes. Strong agglutination is presumptive evidence of a colony being typhoid bacilli.

6. Or place drop of diluted serum on a plain slide and emulsify in each a very small amount of the colonies to be tested. Tilt the slide back and forth for a few seconds over a black background. Agglutination is readily detected by the development of a granular appearance.

Test Tube Method.—This method is recommended for the final identification of typhoid, paratyphoid, and dysentery bacilli as it employs varying dilutions of immune serum.

1. Arrange six small test tubes and place 1 c.c. of distilled water in each.

2. To No. 1 add 1 c.c. of a 1 : 25 dilution of typhoid-immune serum and mix well.

3. Transfer 1 c.c. to No. 2; mix well and transfer 1 c.c. from No. 2 to No. 3 and so on to No. 5, from which 1 c.c. is discarded. The dilutions are 1 : 50, 1 : 100, 1 : 200, 1 : 400 and 1 : 800. No. 6 is the control.

4. To each tube add 1 c.c. of a heavy broth culture of the organism which gives final dilutions of 1 : 100 to 1 : 1600.

5. If the organism is on a solid medium it may be washed off and emulsified in saline solution to give a suspension of approximately 2,000,000 per c.c., and used instead of a broth culture. Or the growth may be removed from an agar slant with a platinum loop and emulsified direct in the serum dilutions; this, however, gives final dilutions of 1 : 50 to 1 : 800.

6. Mix well and place in a water bath at 37° C. for two hours and then in a refrigerator overnight when the readings are made.

7. The same technic may be employed with suitable paratyphoid and dysentery (polyvalent) immune sera.

Absorption Method.—When agglutination tests are indefinite or if cross agglutination occurs, it is possible to identify the organism by absorption tests conducted as follows:

1. Inoculate pint Blake bottles of agar with each culture to be used.

2. After eighteen to twenty-four hours suspend each growth in from 5 to 10 c.c. of saline solution.

3. Centrifugalize each at high speed for thirty to sixty minutes.

4. Remove the supernatant fluids and add 5 to 10 c c of 1:50 typhoid or other immune serum to each sediment.

5. Mix well and place in a water bath at 45° C. for three hours, shaking occasionally, and then in the refrigerator overnight.

6. Centrifugalize at high speed for thirty to sixty minutes.

7. Prepare dilutions of 1:100, 1:200, 1:400, 1:800, and 1:1600 of each supernatant fluid and place 0 5 c.c. of each in small test tubes. In tube No. 6 place 0 5 c.c. of saline solution for control.

8. Add 0 5 c c. of a heavy broth culture or saline suspension of one of the cultures of each tube.

9. Set up duplicate tests with the second or remaining cultures to be tested.

10. Mix well and place in a water bath at 37° C. for two hours and in the refrigerator overnight when the readings are made.

11. If a typhoid serum is employed, the cultures completely absorbing the agglutinin (negative reactions) are of typhoid bacilli.

BACTERIOLOGICAL EXAMINATION OF FECES FOR PARATYPHOID BACILLI IN FECES (SALMONELLA PARATYPHI)

1. Emulsify the material and spread over the surface of Endo plates.

2. Incubate for twenty-four hours.

3. Examine plates for suspicious colonies; paratyphoid bacilli produce bluish-white or colorless colonies.

4. Fish five or ten suspicious colonies and inoculate tubes of Russel's double sugar medium.

5. Incubate the tubes twenty-four hours and examine.

6. If any of the tubes show an unchanged slant and acid and gas in the butt they should be further studied by inoculating litmus milk, glucose and sucrose media.

7. The strains which conform culturally with the genus should be tested by the agglutination test, using immune serum of the *B. paratyphosus A* (*Salmonella paratyphi*) and *B. paratyphosus B* (*Salmonella schotmuelleri*).

8. For certain identification, the stains which agglutinate with either of the immune sera should be tested by the agglutinin absorption test (above).

9. Cultures which fail to agglutinate should be further studied, particularly the fermentation of sugars, for their identification.

BACTERIOLOGICAL EXAMINATION OF FECES FOR B. DYSENTERIAE (SHIGELLA DYSENTERIA)

1. Select mucous flakes from the stool for culturing. The same methods described for paratyphoid and typhoid bacilli should be followed (see above).

2. For identification, agglutination tests should be made, using two immune sera, one for *B dysenteriae* and a polyvalent serum for the paradysenteriae types.

BACTERIOLOGICAL DIAGNOSIS OF CHOLERA (VIBRIO COMMA)

Certain characteristics of the *Vibrio cholerae* are of especial importance because of their practical application in the bacteriological procedure designed for its isolation. These characteristics relate to its morphology (Fig. 220), aerobiosis, and motility; to its marked predilection for alkaline media, to the opalescent appearance of its colony on agar, and to its agglutinability with a specific serum. The following method is recommended for feces:

1. If the specimens are planted in peptone water when collected on board ship or elsewhere, they are incubated six hours.

2. If the stool is sent to the laboratory, inoculate a tube containing at least 20 c.c. of peptone medium with a small amount of feces. Incubate six hours at 37° C. Unless fluid, the desired amount of the specimen should be well rubbed up in a little of the peptone medium before planting.
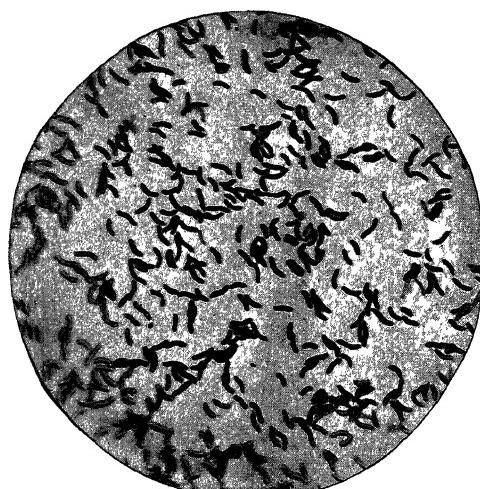
FIG 220—CHOLERA SPIRILLUM.
(After Frankel and Pfeiffer)

3. Plate five loops of the peptone medium culture on a series of three alkaline agar plates.

Make a subculture from the above tubes to tubes of the same medium.

4. Incubate plates and tubes for at least fourteen hours.

5. Examine surface growth of peptone tubes for actively motile vibriones. Conduct indol test for the cholera red reaction.

6. Examine plates for suspicious "heaped-glass" colonies.

(*a*) Test suspicious colonies with agglutinating serum on the slide as follows:

Deposit near one end of a slide a drop of agglutinating serum of a dilution of 1 : 200 (titer not less than 1 : 4000) and near the other end a drop of saline. Now touch the suspected colony with the point of the inoculating needle, rub

up in the drop of saline solution, then flame the point and again *touch* the colony with the point and rub up in the drop of serum solution. Evidence of agglutination will almost instantly be apparent in the latter (if cholera). The drops may be allowed to dry and may be fixed and stained. If agglutination has taken place, it will be evident in the stained specimen to the naked eye, or on slight magnification with the hand lens.

(*b*) If clumping does not occur, test at least ten (and preferably twenty or twenty-five) such colonies, and examine the preparation, after standing, with the microscope for vibriones.

(*c*) If evidence of agglutination on the slide is obtained, or in the event that no agglutination takes place but that the stained preparation shows a vibrio, the colony must be fished and a cholera agar or alkaline plain agar slant inoculated for further study.

(*d*) The crucial test of the specificity of a vibrio is the agglutination test with a serum of high titer.

After incubating the agar slants inoculated with the suspicious colony, or preferably two colonies, for sixteen to twenty-four hours, sufficient culture is on hand for an accurate macroscopic agglutination test in a graded series of serum dilutions.

To be cholera it must agglutinate in a dilution of at least 1 : 1000 (with a serum having a titer of at least 1 : 4000) within two hours at 37° C.

If a vibrio is isolated that fails to do this, repeated daily subculture on agar must be made and its agglutinability tested, for observations are on record showing that occasionally (though rarely) a freshly isolated vibrio may show little or no agglutinability but gain it after a series of subcultures.

If no suspicious colonies are found on the plates make plates from the subcultures (*6c*) which are now fourteen hours old. This series of plates is examined, after incubating at least fourteen hours, in manner precisely like that prescribed for the original set. In the event that suspicious colonies are detected, the procedure to be followed is as described in step 6 above.

If under these circumstances no suspicious colonies should be found, the examination must be regarded as negative.

In this case the procedure will have extended over a period of about thirty-six hours.

Procedure for the Isolation of Vibrio Cholerae from Water.—In this procedure a specimen of about 900 c.c. of the suspected water is desirable, if not necessary.

1. To 900 c.c. of the suspected water add 100 c.c. of "stock" cholera peptone solution, thus converting the specimen into a peptone medium.

2. Distribute this into a convenient number of Erlenmeyer flasks (for example, 200 c.c. in each of five flasks) and incubate at 37° C.

3. The further steps are like those for the isolation from feces, beginning with step 3 of that procedure given above.

BACTERIOLOGICAL DIAGNOSIS OF B. FRIEDLANDER
(KLEBSIELLA PNEUMONIAE) INFECTION

Smears.—1. Prepare smears of pus, sputum or other material.

2. Stain by Gram's method; also by a capsule stain.

3. Examine for gram-negative coccoid bacilli which have a tendency to grow in chains and are usually surrounded with a distinct wide capsule (Fig. 221).

4 Smears alone cannot be depended upon and findings should be confirmed by cultures.

Cultures.—1 Inoculate agar slants or plates (the organism grows well on ordinary media)

2. Examine colonies which are dirty-white, smooth, opaque, glistening and
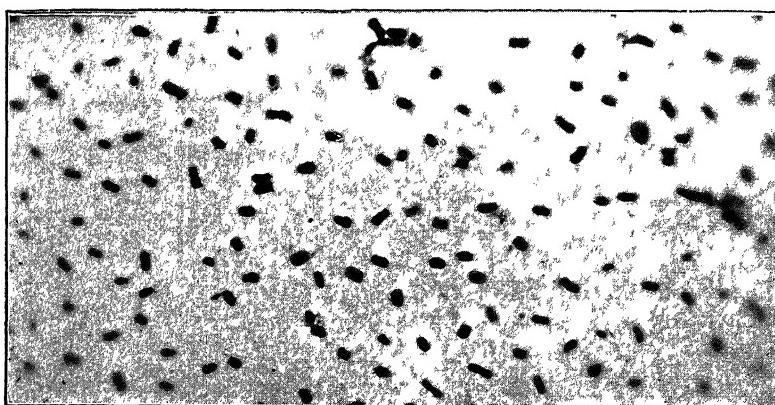
FIG 221 —BACILLUS MUCOSUS-CAPSULATUS   (Zinsser)

mucoid in appearance and are tenacious when removed by platinum loop; stain for gram-negative bacilli which often in cultures show capsule formation.

3 Make subcultures and identify by the four-tube method on page 371.

4 Normally the Friedlander bacillus is occasionally found in the upper respiratory tract and accessory sinuses.

5 In disease it may be the cause of both bronchopneumonia and lobar pneumonia, otitis media, meningitis, pleuritis, pericarditis, and bacteriemia. It may localize and cause abscesses and is occasionally the cause of conjunctivitis.

BACTERIOLOGICAL DIAGNOSIS OF B. PYOCYANEUS
(PSEUDOMONAS AERUGINOSA) INFECTION

1. Inoculate plain agar slants or plates with material to be examined.

2. Incubate at 22° C. for twenty-four to seventy-two hours. The organism grows well at 37.5° C. but pigment production is more marked at the lower temperature. The bacillus is an actively motile slender rod (Fig. 222), fre-

quently united in pairs or in chains of four to six elements and occasionally growing out into long filaments and twisted spirals.

3. Examine for greenish colonies or greenish growth on agar tubes. This coloration is due to the formation of pigments which not only color the growth, but also diffuse through and color the medium. Two pigments are produced: one soluble in water, which is green, and the other soluble in chloroform, which is blue (pyocyanine).

4 To differentiate *B. pyocyaneus* from other fluorescing bacteria, examine an agar culture for pyocyanine by agitating it with chloroform and allow to separate, a blue coloration of the chloroform indicates its presence. The other fluorescing bacteria do not produce pyocyanine.

5 *B pyocyaneus* is widely distributed in air and water, also frequently found in feces. It is commonly found in association with other pathogenic organisms and has been found to be the sole cause of intestinal disorders, middle ear inflammation and cystitis.
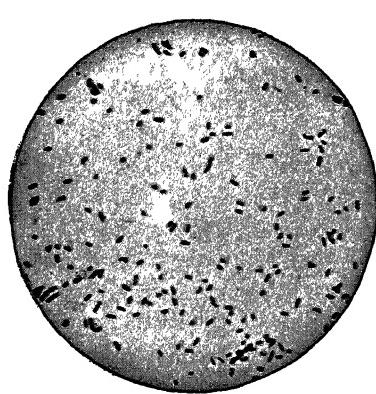
FIG 222—BACILLUS PYOCYANEUS
(From Park, Williams and Krum-
wiede, *Pathogenic Microorganisms,*
Lea and Febiger)
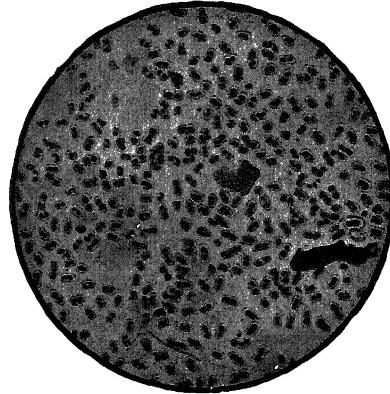
FIG 223—BACILLUS PESTIS
(After Mallory and Wright)

BACTERIOLOGICAL DIAGNOSIS OF PLAGUE

1. Prepare smears of material from bubo or sputum (pneumonic type) and stain by the method of Gram. Gram-negative bacilli which stain more deeply at the poles (Fig 223) suggest *B pestis (Pasteurella pestis)*.

2 If septicemia is suspected, culture 5 to 10 c.c. of blood in a flask of broth medium.

3. Inoculate blood agar (P^H 7 8 to 8 2) plates.

4 Incubate for twenty-four hours. Colonies are grayish-white, translucent, undulant and can be pushed along the surface of the medium.

5 Inoculate tubes of broth for characteristic stalactite formation

6. Inoculate gelatin; no liquefaction by *B. pestis.*

7. Inoculate a guinea-pig subcutaneously and a second one by rubbing

material into the shaved skin of the abdomen. If *B pestis* is present, the animals will usually die in three to five days and show large numbers of bacilli in the blood, liver, spleen, and lymphatic glands.

BACTERIOLOGICAL DIAGNOSIS OF GLANDERS

Cultures.— 1. *B. mallei* (*Pfeifferella mallei*) occurs in the nasal discharge and lesions of the skin and organs.

2. Inoculate glycerin agar or blood serum and glycerin potato. The media should be slightly acid in reaction.

3. Incubate at 37.5° C   At the end of twenty-four hours a growth appears.

4. The colonies are moist, grayish-white, translucent, and ropy.   On potato the growth is usually more abundant and at first is pale yellow and honey-like; later it assumes a reddish-yellow or chocolate color.

FIG 224—GLANDERS BACILLUS
(Zinsser)

5. Make smears of the cultures and stain by Gram's method and with methylene blue.

6. The organisms do not stain well and often show irregular staining and some appear to contain granules.   They are slender and often slightly curved, usually single, in pairs or groups (Fig. 224).

7. Culture can be definitely identified by agglutination test with immune serum, although freshly isolated strains are not easily agglutinated.

8. In conducting this test, heat several forty-eight-hour glycerin-agar slant cultures at 60° C. for two hours and suspend the growths in saline solution carrying 0.3 per cent tricresol.   Shake well for a homogeneous suspension and filter if necessary through soft paper.

Set up agglutination tests with 0.5 c.c. of varying dilutions of a known immune serum and add 0.5 c.c. of the bacterial suspension.   Include saline controls and preferably a set of controls employing normal serum.

Incubate at 37° C. for twenty-four hours and read the reactions.

Inoculation of Animals (Strauss Test).—1. Inject intraperitoneally a male guinea-pig with a small amount of suspected material.

2. If *Pfeifferella mallei* is present, an orchitis will develop in three to four days.

3. As soon as the orchitis is well developed, destroy the animal and culture the testes as just described.   If the animal is allowed to live, the testes will

abscess and discharge. Lesions will also develop in the liver, spleen, pancreas, lungs, etc.

BACTERIOLOGICAL DIAGNOSIS OF HEMORRHAGIC SEPTICEMIA
(PASTEURELLA BOVISEPTICA AND SUISEPTICA)

Cultures.—1. At the height of the infection the blood may contain the organisms. After death the blood and affected organs should be cultured.

2. Streak or spread the material on blood agar plates.

3. Incubate at 37° C. for twenty-four hours.

4. Examine for small, transparent, slightly raised, discrete colonies. Subculture in infusion broth.

5. Incubate for twenty-four hours and examine smears stained by Gram's method and with methylene blue. The bacilli are small, gram-negative, and stain more intensely at the ends (bipolar staining).

Animal Inoculation.—1. Inject 1 c.c. of broth culture or tissue emulsion intravenously or intraperitoneally into rabbits.

2. If *Pasteurella boviseptica* or *suiseptica* is present, the animal will die in from one to four days.

3. At autopsy the characteristic lesions are: slightly swollen spleen, petechial hemorrhages of the serous membranes, and marked laryngotracheitis.

4. Make smears and cultures of blood and organs.

Pasteurella boviseptica causes hemorrhagic septicemia of cattle, hogs, and horses.

Pasteurella suiseptica is the cause of swine plague.

BACTERIOLOGICAL DIAGNOSIS OF FOWL CHOLERA
(PASTEURELLA AVISEPTICA)

Smears.—1. Make thin smears of blood and tissues.

2. Stain with methylene blue and by Gram's method.

3. Morphological and staining characteristics of the bacillus are the same as for *Pasteurella boviseptica* (see above).

Culture and Animal Inoculation.—Same as described for hemorrhagic septicemia (see above).

BACTERIOLOGICAL DIAGNOSIS OF UNDULANT FEVER
(ALCALIGENES MELITENSIS AND ABORTUS)

Cultures.—1. Collect blood for blood cultures. If exudates containing other organisms are to be cultured it is advisable to plate or inoculate guinea-pigs.

2. Inoculate several tubes or plates of liver infusion agar and agar containing serum and nutrose.

3. Divide the cultures into two sets and incubate one at 37° C. under ordinary aerobic conditions and the second in glass jars in which at least 10 per

cent of the air has been replaced with carbon dioxide for lowered oxygen tension.

4. Examine cultures daily.

5. Growth is slow and may require several days to a week or two before it appears.

6. The colonies are minute, clear, round, and slightly raised. Subcultures of suspicious colonies should be made on the same kind of media and incubated under the same conditions. Subsequent subcultures grow more rapidly and luxuriantly and after a few transplants aerobically.

The organism is very small, often so short as to resemble cocci. It is quite pleomorphic, and stains well with all aniline dyes.

7. Identify by agglutination tests with immune serum.

Differentiation between Alcaligenes Abortus and Alcaligenes Melitensis. —1. This is rather difficult inasmuch as the two organisms are very similar in their morphological, staining, and cultural characteristics.

2. They cross-agglutinate and therefore agglutination tests are of little value.

3. Agglutinin absorption tests are recommended and to some extent will differentiate between the two.

4. Primary cultures of *Alcaligenes abortus* will not grow under aerobic conditions (required partial anaerobiosis), whereas primary cultures of *Alcaligenes melitensis* will grow under ordinary aerobic conditions.

Animal Inoculation.—1. Inoculate guinea-pigs with suspected material subcutaneously.

2. At the end of six to eight weeks kill and examine the animals.

3. Look for tubercle-like lesions in the spleen, liver, lungs, and kidneys.

4. Make smears and stain for acid-fast bacilli to eliminate tuberculosis.

5. Culture as described above.

6. This method is useful as a means of isolation from material contaminated with other organisms.

Alcaligenes melitensis causes undulant fever in man, sheep and goats.

Alcaligenes abortus has been found to be the cause of sporadic cases of undulant fever in man. It is the cause of bovine and porcine infectious abortion.

BACTERIOLOGICAL DIAGNOSIS OF INFECTIONS WITH ALCALIGENES BRONCHISEPTICUS

1. Material such as nasal or bronchial secretions should be plated on plain or blood agar.

2. Incubate at 37° C.

3. Growth usually occurs in twenty-four hours. The colonies are small, round, slightly raised, and pin point in size.

4. Subculture suspicious colonies into tubes of litmus milk.

5. Incubate the milk cultures for twenty-four hours and then discard any which show signs of acid production. The others should be studied to determine if they belong to the genus Alcaligenes.

6. Agglutination tests with immune serum can be used for identification.

Alcaligenes bronchisepticus causes "snuffles" in rabbits, pneumonia in guinea-pigs, and is found as a secondary infection in distemper of dogs.

BACTERIOLOGICAL EXAMINATION OF BILE AND DUODENAL FLUIDS

1. Two c.c. of fluid are drained directly by a special technic into flasks containing broth (see Chapter X). The medium should be favorable for the growth of streptococci and special precautions taken to prevent contamination from the upper digestive tract.

It is well to stress the difficulty of isolating streptococci in the presence of other rapidly growing organisms, particularly the colon bacillus; when streptococci are seen in fresh smears a special effort should be made to get them out, as the streptococci are of immense importance clinically.

2. Incubate the flasks for twenty-four hours. Should no growth occur, continue the incubation for two or three days.

3. Make smears of the broth and examine after staining by Gram's method.

4. If the culture appears to contain only one organism, make subcultures on blood-agar slants. If more than one organism appears to be present, make blood-agar plates.

5. Isolate and identify organisms present.

BACTERIOLOGICAL DIAGNOSIS OF SYPHILIS

1. *Spirochaeta pallida* (Fig. 225) may be found in primary and secondary lesions by dark-field examination (recommended) or by special staining methods.

2. The technic of these is described on page 314.

3. Cultures are not employed.

4. Secretions, bits of tissue, spinal fluid and material aspirated from en-
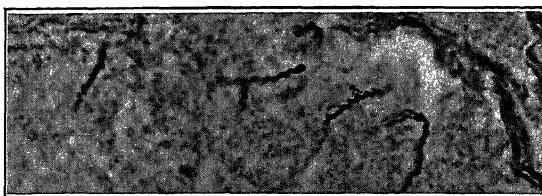
FIG. 225.—SPIROCHAETA PALLIDA; LEVADITI METHOD.   (Zinsser.)

larged lymph glands may be inoculated into the testicles of rabbits. Full-grown and healthy animals should be employed. From 0.2 to 1.0 c.c. of fluid or emulsion should be injected into the center of each testicle with a sterile

syringe after sterilization of the skin with iodine. Syphilitic orchitis develops in two to six weeks with numerous spirochetes to be seen by dark-field examination. Chancres of the scrotal skin may appear.

BACTERIOLOGICAL DIAGNOSIS OF RECURRENT FEVER

1. *Borrelia recurrentis* (*Spirochaeta obermeiereri*) and *Borrelia novyi* (*Spirochaeta novyi*) may be found in blood films fixed with methyl alcohol and stained with diluted carbolfuchsin or after staining with Jenner's or Wright's blood stains (Fig. 226). Careful search for the spirochetes is usually required.

FIG 226—BORRELIA RECURRENTIS.
(After Calkins)

2. Cultures are not employed.

3. From 0 2 to 0.5 c.c. of patient's blood or clots broken up in sterile saline solution may be injected intraperitoneally into a white mouse under aseptic precautions.

4. Examine a drop of blood from the tail on a slide covered with cover glass with high dry or oil-immersion lenses each day over a period of at least five to fourteen days for spirochetes. Infected mice are likely to survive for months with recurrent spirochetemia.

BACTERIOLOGICAL DIAGNOSIS OF SPIROCHETIC AND AMEBIC GINGIVITIS

1. Wet and stained preparations are recommended.

2. The material should be collected with care, especially from gingival pockets with suitable instruments or after expression by pressure.

3. Prepare several smears. Dry in the air. Fix with heat. Stain with 1:10 carbolfuchsin for two or three minutes. Wash with water, dry, and examine with oil-immersion lens for spirochetes (*Spirochaeta microdentium; Spirochaeta macrodentium,* etc.).

4. An occasional spirochete is normal. But large numbers and tangled masses represent a pathological increase (spirochetic gingivitis).

5. Fusiform bacilli may also be found in association with the spirochetes (*Spirochaeta vincenti*) constituting spirofusillar gingivitis, a form of Vincent's angina infection of the gums, or trench mouth.

6. A few spirochetes and fusiform-shaped bacilli resembling *B. fusiformis* are to be found in most mouths and do not alone constitute evidences of infection; but the presence of large numbers is regarded as pathological.

7. *Leptotrichia buccalis* is frequently found in the mouth and may be mistaken for fusiform bacilli. They occur as long, gram-positive bacilli or filaments. They do not grow under ordinary aerobic conditions.

8. Wet preparations are especially desired for examination for *Endamoeba gingivalis* (Gros).

9. Warm a slide. Place a drop of warm saline solution. Add a small amount of gingival secretion. Cover with cover glass and examine for motile amebae with high dry lens and with the light well reduced.

10. Spirochetes may be likewise detected in these preparations or by darkfield examination.

11. An occasional ameba may be found in the absence of gingivitis. One or more per field, however, represents an increase and may produce gingival infection by opening up avenues of bacterial infection.

BACTERIOLOGICAL DIAGNOSIS OF INFECTIOUS JAUNDICE

Smears, Cultures and Animal Inoculation.—1. Examine for *Leptospira icterohaemorrhagiae* in the urine and blood.

2. Urine may be centrifuged and the sediment examined by the dark-field method.

3. Catheterized urine is suitable for cultures. Inoculate tubes of the rabbit-serum of Noguchi with several loopfuls of sediment and cultivate anaerobically.

4. Inject white guinea-pigs (8 to 10 ounces) intraperitoneally with sediment suspended in saline solution (same technic as inoculation for tubercle bacilli).

5. Inoculate white guinea-pigs of same weight intraperitoneally with 0.5 to 1 c.c. of citrated blood or with clots broken up in sterile saline solution, taken during the first week of the disease.

6. Inoculate rabbit-serum medium with o 5 c.c. of blood and cultivate anaerobically.

7. Incubate all cultures at 25° C. and examine each weekly by dark-field for at least four weeks for motile leptospirae.

8. Examine the guinea-pigs daily for jaundice of the skin and sclerae. Take temperature daily. When a marked rise occurs, remove 1 or 2 c.c. of blood from the heart in an equal amount of 1 per cent solution citrate solution. Examine by dark-field for leptospirae. If organisms are found, chloroform the animal Look for jaundiced tissues and numerous petechial (butterfly) hemorrhages, especially in the lungs and inguinal region.

9. Prepare sections of the liver and kidneys to be stained for leptospirae.

Agglutination and Lysis.—1. If a culture is available, prepare equal parts of o 5 c.c. of culture and 1 : 2, 1 : 4, 1 : 8, 1 : 16, and 1 : 32 dilutions of patient's serum in small test tubes. Place in water bath for two hours. Examine each and a control for agglutination by dark-field.

2. In a test tube place 0.5 c c. of culture (upper portion), 0.5 c.c. of the patient's fresh unheated serum and 1.5 c.c. of saline solution. Mix and inject

the whole into the peritoneal cavity of a guinea-pig. At intervals of fifteen minutes withdraw a small amount with fine capillary tubes and examine by dark-field for evidences of agglutination and lysis.

3. Inject a second pig with a control mixture, using normal human serum.

4. If antibodies are present, agglutination with partial or complete lysis usually occurs within an hour.

BACTERIOLOGICAL DIAGNOSIS OF RINGWORM

1. Examine hairs in the same manner as described under favus (see below).

2. There are three different species of fungi causing ringworm:

Trichophyton megalosporon endothrix: the cause of ringworm of the beard (barber's itch). It is composed of spores of various sizes lying in chainlike formation up and down the hair shaft and mycelium which are not found within the hair (Fig. 227).

FIG 227 —HAIR RIDDLED WITH RINGWORM FUNGUS, MEGALOSPORON VARIETY.
(From Park, Williams and Krumwiede, *Pathogenic Microorganisms,* Lea and Febiger)

Trichophyton microsporon: the cause of ringworm of the scalp. It differs from the preceding as follows: spores smaller and round, irregularly grouped around follicular portion of hair, mycelia often found within the hair.

Microsporon furfur: the cause of the condition known as tinea versicolor. It is composed of spores arranged in clusters which are highly refractive and resemble droplets of oil. The mycelia are not usually branched. Found readily in the scales scraped from the affected skin. Stains better than the favus or other ringworm fungi.

BACTERIOLOGICAL DIAGNOSIS OF FAVUS

1. Macerate a favus crust with a few drops of water or, better, a 10 per cent solution of sodium or potassium hydroxide.

2. Place material on slide, cover with cover glass and gently press out. Hairs from affected areas should be examined in the same manner.

3. Examine with low and high dry objectives.

4. The fungus (*Achorion schoenleinii*) appears as threads (mycelia) which may be simple or segmented, and as irregularly shaped bodies (conidia) which are in chains or encapsulated.

Staining Method for Favus and Ringworm Fungi.—1. Material collected by scraping the skin should be mixed with egg albumin or serum and smeared on slide and dried.

2. Treat with alcohol and then with ether (to reduce fatty material).

3. Cover with Gram's iodine for one to two minutes.

4. Dry.

5. Stain with aniline gentian violet for one to five minutes.

6. Dry in air.

7. Cover with Gram's iodine for one or two minutes.

8. Clear with aniline oil.

9. Wash in xylol.

10. Mount in balsam or lactophenol (phenol, 20 c c.; lactic acid, 20 c.c.; glycerin, 40 c c.; water, 20 c.c.).

Cultural Method for Favus and Ringworm Fungi.—1. Inoculate Sabouraud's maltose or Weidman's dextrose agar slants or plates. Do not seal tubes or place rubber caps over mouth. The reaction of the media should be about $+2$ (P^H about 5.0).

2. Incubate at 30° to 36° C.

3. Growth may not become visible until after one week or even longer. The culture is seldom pure and subsequent transplantations have to be made to obtain pure cultures.

BACTERIOLOGICAL DIAGNOSIS OF BLASTOMYCOSIS

Smears.—1. Prepare smears on slides or cover glasses.

2. Stain with methylene blue or Jenner's stain.

3. Examine for yeast cells occurring in torular form (budding).

Cultures.—1. Blastomycetes grow well on ordinary media with reaction neutral, slightly acid, or alkaline.

2. Inoculate agar slants or plates.

3. Incubate for a week or ten days.

4. Examine for rather large cells resembling yeast cells and multiplying by budding.

BACTERIOLOGICAL DIAGNOSIS OF SPOROTRICHOSIS

Direct Method.—The method outlined for favus may be used.

The material for examination should be collected from the nodules or the pus if discharging.

The direct method is not very satisfactory, as the organisms can rarely be demonstrated by this method. Cultural method should always be employed.

The fungus appears as highly refractile conidia (spores) which are gram-positive and may be seen in tissue cells.

Cultures.—1. Inoculate material on Sabouraud's or Weidman's dextrose agar.

2. Growth appears in two to seven days, as small white colonies which later become larger, circular, and characteristically raised and ringed.

3 Examine unstained preparations of suspicious growth.

4. The fungus appears as narrow filaments with spores at the sides and ends without definite fruiting organs.

BACTERIOLOGICAL DIAGNOSIS OF ACTINOMYCOSIS

Direct Examination of Pus.—1. Spread material over the bottom of a Petri dish and look for small white or yellowish "sulphur" granules about the size of a pin head (Fig. 228). These granules are colonies of actinomyces.

2. By means of a platinum loop place a granule on a slide and cover with cover glass; press out gently.

3. Examine with 2/3 or oil-immersion lens. The actinomycotic granule
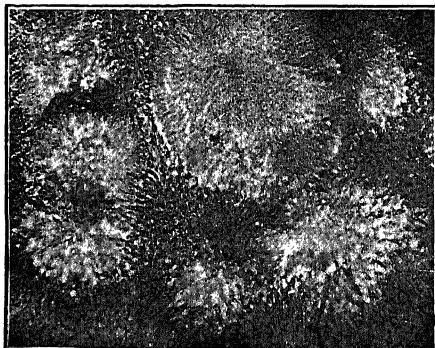
FIG 228 — ACTINOMYCES GRANULE
CRUSHED BENEATH A COVER GLASS.
(After Wright and Brown.)
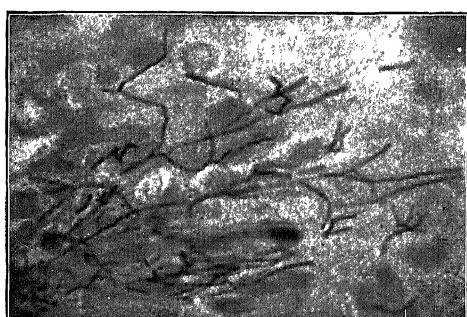
FIG. 229 —BRANCHING FILAMENTS OF
ACTINOMYCES
(After Wright and Brown)

(colony) will be made up of filaments radiating from the center (Fig. 229). The terminal ends of the filaments around the periphery will appear bulbous or club-shaped, giving the appearance of rays of light radiating from a center (ray fungus).

Stained Smears.—1. Prepare thin smears of material, particularly the granules just described.

2. Stain with Gram's stain. The central mycelium of the colony will stain gram-positive; the clubs or bulbous ends will not stain with Gram's.

3. Also stain smears by acid-fast method, as some strains are acid-fast.

Cultures.—1. Inoculate eight or ten tubes of glucose agar or blood serum with crushed granules. Sabouraud's agar is recommended.

2. Incubate one half at 37° C. aerobically and the remainder anaerobically.

3. Examine after four or five days. At first the colonies are very small, dry, chalky, and adherent.

4. Prepare smears and stain by Gram's method. The organism is gram-positive and appears as long filaments with true branching.

5. Some strains grow only anaerobically.

6. If no growth occurs in two weeks, the organisms are absent.

7. If the material contains many bacteria it may be dried for several weeks and then cultured. The nonsporing bacteria are apt to die off while the actinomyces survive.

BACTERIOLOGICAL DIAGNOSIS OF ERYSIPELOID OF MAN AND SWINE ERYSIPELAS

1. Erysipeloid (Rosenbach) of man and "swine erysipelas" are caused by *Erysipelothrix rhusiopathiae*. The disease may occur among handlers of fish (Klauder and Harkins).

2. Excise pieces of skin about 3 millimeters square from infected areas; grind in a sterile mortar and inoculate tubes of hormone broth. Cultivate aerobically.

3. Inoculate mice intraperitoneally or pigeons intramuscularly with 0.2 c.c. of two- to four-day cultures; pure cultures may be obtained from the blood of the heart; also by plating on hormone blood agar.

4. The organism occurs as a gram-positive coccoid or short, straight or slightly curved bacillus. Old cultures on agar are threadlike.

BACTERIOLOGICAL DIAGNOSIS OF THRUSH

1. Remove a small portion of white patch from mucous membrane

2. Press out well between a cover glass and slide.

3. Examine with 2/3 and higher power objectives.

4. The thrush fungus *Endomyces (Monilia) albicans* appears as a network of filaments which may be segmented and branching or as yeast-like forms shining budding. Epithelial cells, leukocytes, and débris will also be present (Fig. 230).
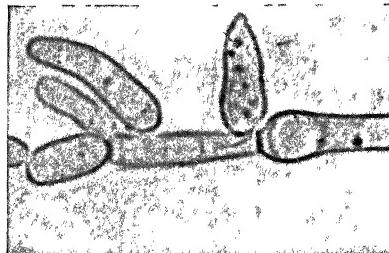
FIG. 230.—OIDIUM ALBICANS.
(After Zettnow.)

BACTERIOLOGICAL DIAGNOSIS OF ASPERGILLOSIS

Direct Method.—1. Mount the material scraped from the lesions by the method described for ringworm and favus.

2. Examine for mycelia showing fructification at the end in the case of the violet or gray-violet varieties, and filaments and scattered spores in the case of the white, black and red varieties.

Cultures.—Employ same method as described for culturing favus and ringworm.

LABORATORY DIAGNOSIS OF RABIES

Smears of Brain Tissue.—1. When removing the brain from the animal (usually a dog) wear heavy rubber gloves. It is also advisable to wear goggles.

2. Be sure to boil immediately the instruments and wipe up the table with 10 per cent formalin solution.

3 Prepare smears from the cornu ammonis (hippocampus major), the cerebral cortex near the Rolandic fissure and cerebellum. The best results will be obtained by making impression smears by first removing a section of the hippocampus, making a smooth cut across one end Pick up with forceps, holding it near cut end. Bring cut surface in contact with clean slide, press lightly against slide and then remove Sufficient material adheres to slide for examination. Make several of these impressions on the same slide (six or eight)

4 Place slides in methyl alcohol for two or three minutes.

5 Remove from alcohol and allow to air dry.

6. Cover smears with the following stain and steam gently:

Basic fuchsin (sat. alc sol)	3 drops
Loffler's methylene blue	2 c c.
Water (distilled)	10 c c.

7. This stain will keep fairly well in ice chest.

8. Wash with water and dry

9. Locate ganglion cells with low dry objective and then examine the cells with oil immersion for the Negri bodies, which appear as round or oval bodies, stained magenta and containing blue granules (Plate IX). Nerve cells, blue; red cells are salmon or yellow.

10. Place excess tissue in sterile, neutral, C P. glycerin, using 3 parts of glycerin to 1 of tissue. Keep in refrigerator for later use if necessary If the brain is decomposed, leave the material in glycerin for at least three days before using for animal inoculation

Tissue Sections.—1. This method is used when direct smears fail to show Negri bodies.

2 Place pieces not over 1 by 1 by 0 2 centimeter in size in 10 parts of Zenker's fluid for eight hours.

3. Wash in running water for eight to twenty-four hours.

4. Place in 80 per cent alcohol for one hour and then in 95 per cent absolute alcohol for one hour each.

5. Place in xylol for one hour and then in xylol-paraffin and paraffin in the incubator for one hour each.

6. Imbed in paraffin in the usual manner and cut thin sections.

7. Stain by Mallory's or Goodpasture's methods.

Animal Inoculation.—1. Macerate small portions of brain tissue in salt solution.

PLATE IX
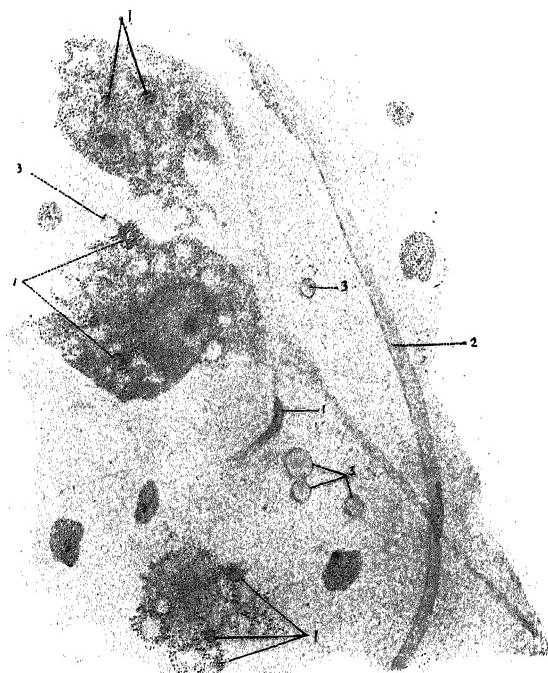
NERVE CELLS CONTAINING NEGRI BODIES.

Hippocampus impression preparation, dog. Van Gieson stain. × 1000.
1, Negri bodies; 2, capillary; 3, free red blood corpuscles. (From Todd
and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

2. Inoculate a rabbit subdurally with a few drops of the brain emulsion.

3. In positive cases the animal will develop symptoms of rabies in the dumb form (paralysis) in about three weeks. It is, however, necessary to keep the animals under observation for sixty to ninety days before reporting negative results.

4. If the animal develops symptoms and dies, the brain should be removed and examined for Negri bodies.

5. If the animal dies within six days, death is probably due to bacterial infection and not to rabies. Repeat, using glycerinated material.

LABORATORY DIAGNOSIS OF KALA-AZAR

1. Examine the blood for anemia due to reduction of erythrocytes and hemoglobin (color index about normal); leukopenia with reduction of polymorphonuclears and eosinophils with increase of lymphocytes and monocytes.

2. Stain smears of blood and examine the polymorphonuclears and monocytes for *Leishmania donovani*; the parasites may rarely occur in erythrocytes. Thick films may be prepared, dehemoglobinized, and stained according to the methods described in Chapter V for the detection of malarial parasites. Wright's or the Giemsa stain may be employed.

3. Blood cultures are usually positive. From 0.25 to 0.5 c.c. of blood removed aseptically from a vein at the elbow is placed in 20 c.c. of sterile 1.5 per cent sodium citrate in physiological saline solution. The mixture is gently shaken and allowed to stain overnight in a refrigerator or centrifuged. The supernatant fluid should be decanted and the corpuscles transferred with a sterile pipet to the water of condensation in tubes of N.N.N. medium (1.5 per cent agar with 0.25 per cent glucose and containing about one-third volume of sterile rabbit blood added to the medium at 52° C.). Incubate at 22° to 24° C. (room temperature). Smears are stained with the Wright or Giemsa stains for flagellates.

4. Cultures and smears of the spleen may be made by aspiration with a sterile syringe and are valuable diagnostic aids. Aspiration of the liver is stated to be a safer procedure but frequently yields negative results. Smears of material obtained from bone puncture, excised lymphatic glands, scrapings from bases of artificially produced blisters or ulcers, are stated by Brahmachari to be unsatisfactory.

5. Leishman-Donovan bodies (see Plate IV) as seen in cells are circular to elliptical in shape, from 2 to 4 micra in diameter, and contain two nuclei, a large oval one at one part of the periphery and a small circular or rod-shaped one (blepharoplast) near or at the opposite part of the periphery (Fig. 231). This smaller body stains more deeply than the larger one, while the cytoplasm of the parasite stains very dimly, sometimes showing only a faint peripheral rim. Flagellates are seen in cultures (Fig. 232).
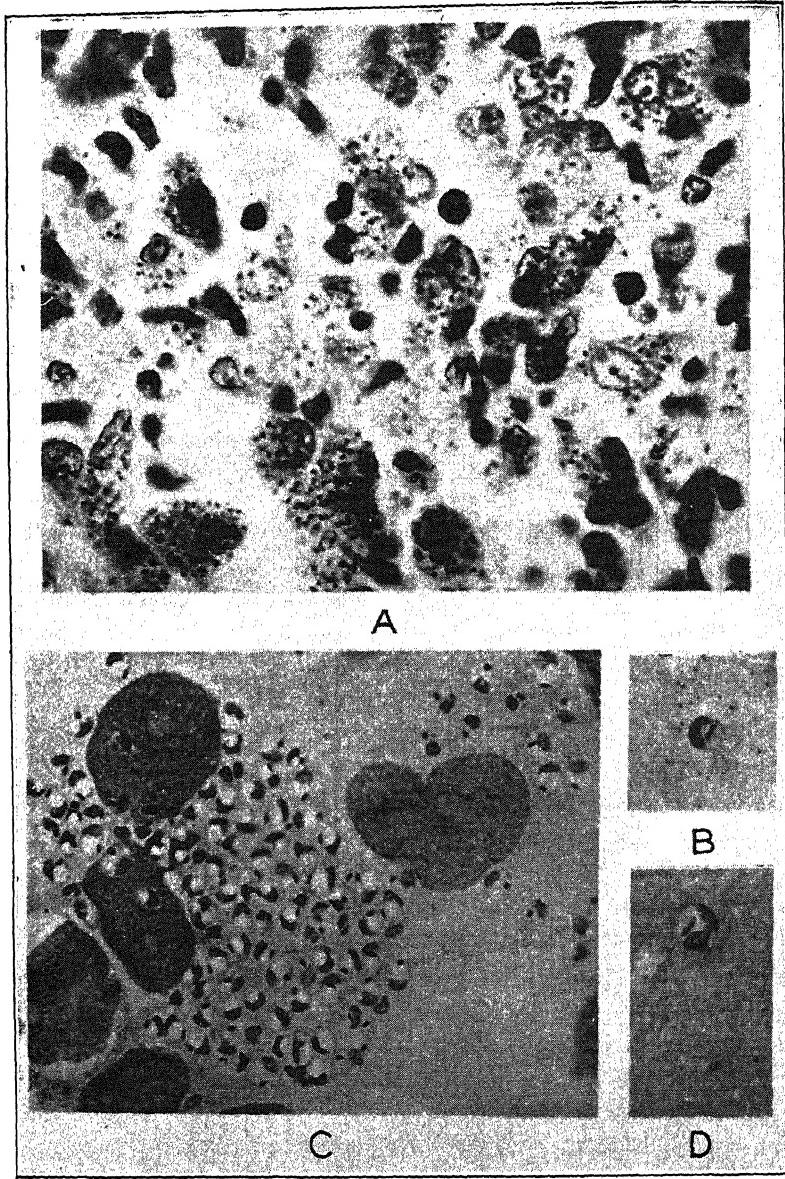
FIG. 231.—LEISHMANIA TROPICA.

A. Section of cutaneous lesion showing organisms included in large mononuclear cells. *C.* A large mononuclear cell showing cytoplasm filled with organisms in film stained with Wright's stain. *B* and *D.* Dividing forms, Wright's stain. (After Wright.)
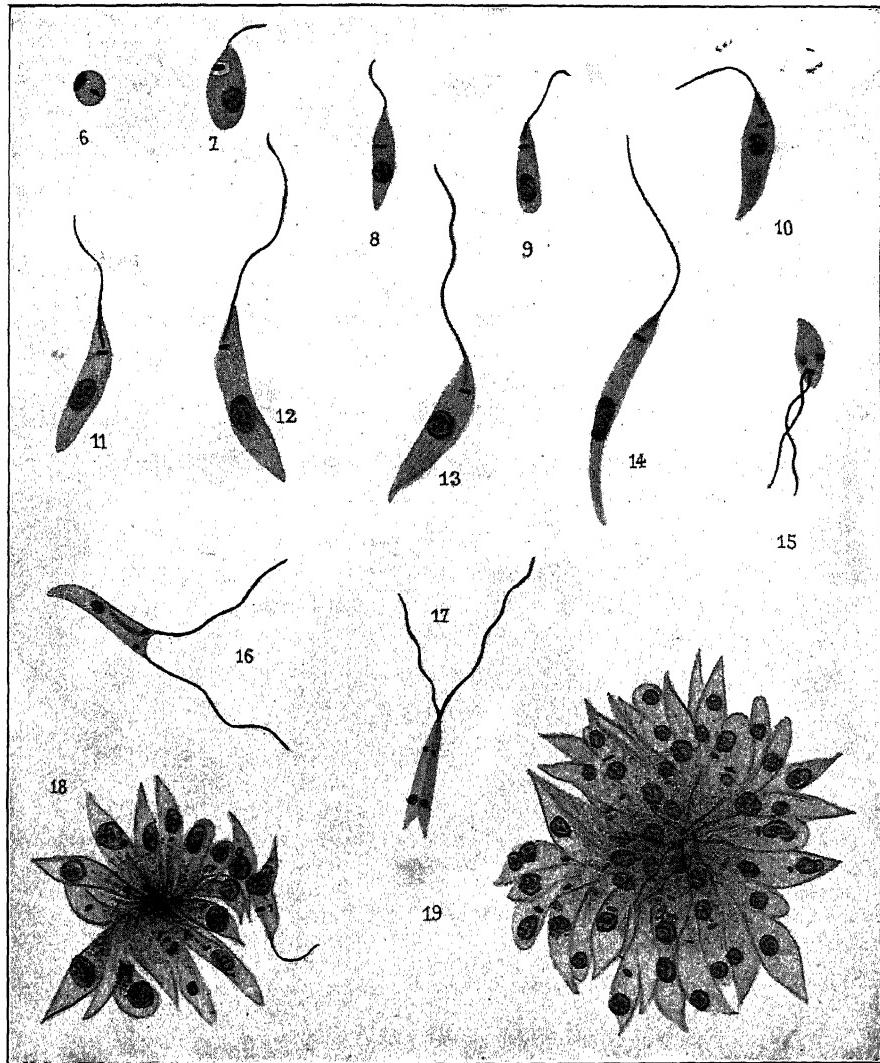
FIG. 232.—CULTURAL FORMS OF LEISHMAN-DONOVAN BODIES.
(From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

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LABORATORY DIAGNOSIS OF GRANULOMA INGUINALE

1. The diagnosis is dependent upon the finding of "Donovan-like" bodies, very similar to those described above, in the endothelial cells obtained by scraping the granulating surface of the ulcerations.

2. Carefully cleanse the surface of the ulcer with saline solution to remove any exudate which may be present.

3 Scrape the surface with the edge of a scalpel to obtain tissue cells. Blood and pus should be avoided as much as possible. The procedure may be painful and it may be necessary to anesthetize the surface with a few drops of novocaine solution. The scrapings should be deep enough to include many tissue cells (endothelial cells) as superficial scrapings or material on the surface are usually unsatisfactory.

4. Prepare smears of the scrapings and stain with Wright's or Giemsa stains.

5. Examine the endothelial cells present for small oval bodies which are found within the cytoplasm of these cells (see Plate IV). These bodies are described as "Donovan" bodies, which they resemble very closely.

CHAPTER XX

METHODS FOR THE PREPARATION OF BACTERIAL VACCINES

GENERAL PRINCIPLES

1. The therapeutic activity of bacterial vaccines is ascribed to a combination of specific and nonspecific effects. Prophylactic immunization depends mostly upon specific effects.

2. Specific effects depend upon the production of antibodies, mostly antitoxins and opsonins.

3. Different species of bacteria vary greatly in vaccinogenic activity. As a general rule, freshly isolated strains are more antigenic than stock strains.

4. The soluble or exogenous toxins of pathogenic bacteria are particularly desirable in vaccines because of their superior vaccinogenic activity.

5. Owing largely to the existence of immunologically specific strains of some bacteria, autogenous vaccines are to be preferred to stock vaccines in the treatment of disease to secure the maximum of specific effects.

6. However, a well-prepared stock may be superior to a poorly prepared autogenous vaccine due to overheating or in which the important organisms are missing, etc.

7. Some vaccines must be of stock cultures owing to technical difficulties in securing the organisms from individual cases, as in the case of chronic gonorrhea and tuberculosis. Vaccines for prophylactic immunization are stock vaccines.

8. The first principle in preparing an autogenous vaccine is to make cultures in a proper manner to secure the bacteria of primary importance rather than the organisms of only secondary infection; technic and choice of culture media are of importance.

9. In mixed infections judgment must be used in selecting the organisms to be incorporated into the vaccine. Spore-bearing bacilli like *B. subtilis* should be always excluded; likewise such contaminating bacteria as *B proteus-vulgaris, B prodigiosus*, etc.

10. The pathogen selective method (given below) may be helpful in selecting bacteria for vaccines in mixed infections on the basis that only those capable of growing in the whole coagulated blood of the patient are apt to be virulent. It is possible, however, that an organism unable to grow in the blood or produce septicemia may still be able to produce local infection.

11. Intracutaneous skin tests are also advocated for the selection of bacteria in the preparation of vaccines on the principle that only those yielding positive reactions should be employed. Positive skin reactions may be an indication of acquired allergic sensitization and therefore evidence of infection with the organism; they may be also plain inflammatory reactions if the inoculum contains a toxin or toxins for which there are no or insufficient amounts of antitoxin in the blood. The exact value or status of the method is as yet unknown.

PREPARATION OF CULTURES

1. As stated above, the method employed for making cultures is very important. The proper technic and culture medium should be employed. Blood agar is a good routine medium although hormone-glucose broth or the Rosenow brain broth medium are to be preferred when streptococci or pneumococci are suspected. The preliminary examination of a stained smear of pus or other material is advised and aids in choosing the proper medium.

2. Incubate cultures for twenty-four to forty-eight hours and examine by smears stained by Gram's method.

3. If there is more than one kind of organism, secure each in pure culture by plating.

4. Cultivate each on two or three tubes of a solid or in a broth medium for twenty-four hours or longer as necessary to secure sufficient organisms for the preparation of vaccine.

5. Examine each culture by stained smear for purity.

PREPARATION OF SUSPENSIONS

1. If a solid medium is used, cover the growth on one of the tubes with sterile salt solution, taking precautions against contamination. Bring the organism into suspension either by shaking or with a platinum loop (Fig. 233).
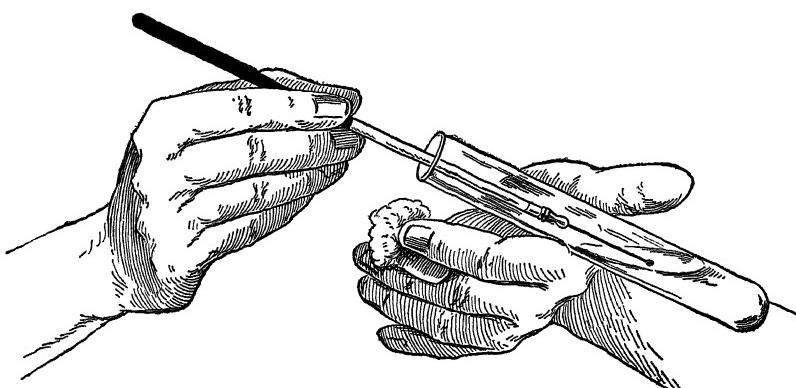
FIG. 233.—PREPARATION OF A BACTERIAL VACCINE.
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

2. The suspension from the first tube should be transferred to the second tube and the growth removed in the same manner. Repeat the process until the growths from all tubes have been suspended.

3. If bouillon is used, the culture should be centrifuged at high speed, the bouillon removed and the sediment containing the bacteria resuspended in salt solution. If the bouillon contains other than human serum, it should be again centrifuged and resuspended in salt solution.

4. The suspension prepared in either manner should be quite heavy and equal in density to at least tubes 5 to 8 of the nephelometer.
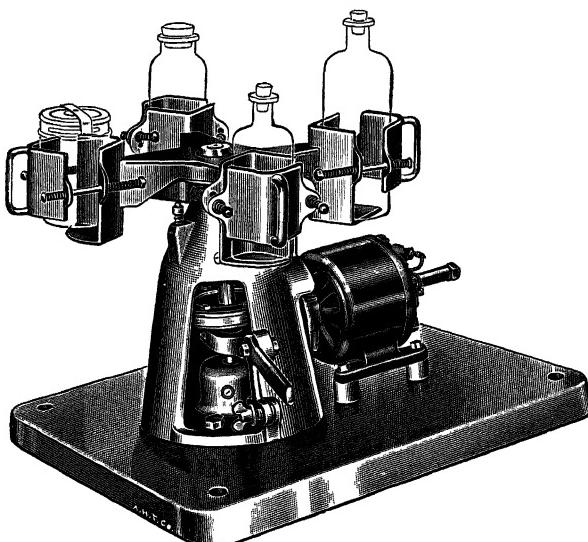
FIG 234.—MECHANICAL SHAKER FOR VACCINES.

5. Transfer the suspension to a sterile flask or bottle containing sterile beads and shake well to break up clumps (clumps of staphylococci and typhoid bacilli are easily broken up; streptococci, pneumococci and diphtheroids require more shaking). A mechanical shaker is recommended (Fig. 234).

6. Filter the suspension through sterile paper (it is convenient to have on hand small funnels with folded paper in place wrapped in newspaper and sterilized in hot air oven).

STANDARDIZATION OF VACCINES

1. As a general rule, vaccines for administration to adults may contain a total of approximately 1,000,000,000 organisms per c.c. For children, one-half this strength may be used.

2. A dose of 0.1 c.c. of such vaccines will carry 100,000,000 and 50,000,000 respectively, which are ordinarily satisfactory for the first injections. It is

possible, however, to give 0.05 c.c. with a tuberculin syringe if a smaller initial dose is desired. Subsequent doses may be increased by 0.1 or 0.2 c.c. as desired.

3. If two or more organisms are to be used, a suspension should be prepared of each containing 1,000,000,000 per c.c. (adult). After sterilization, *equal* parts of these individual vaccines may be mixed to make one vaccine.

Counting Chamber Methods (Recommended).—1. In a small sterile test tube place 0.1 c.c. of suspension and 4.9 c.c. of sterile saline solution (gives a 1:50 dilution); use sterile pipets. Mix well.

2. Draw the suspension up to the 0.5 mark in a leukocyte-counting pipet.

3. Draw the following stain up to the 11 mark (gives 1:20 dilution):

Crystal violet (sat. alc. sol.)............. .. 10 c.c.

Water (freshly distilled)... ... . 100 c.c.

Filter a small portion immediately before using.

4. Slip a wide rubber band over the ends of the pipet and shake for at least two minutes. Do not allow the dilution to stand.

5. Discard a few drops on a piece of filter paper (discard into a disinfecting solution) and then place a drop in the center of a Helber counting chamber (thoroughly cleaned to avoid dust particles).

6. Accurately adjust a reinforced precision cover glass.

7. Allow to stand for fifteen minutes.

8. With a No. 6 objective and No. 4 eyepiece make a count of the bacteria in at least 20 squares, being careful to focus on different levels for bacteria that have not settled.

9. If the number of bacteria is too large for a fairly accurate count (the number per square should be within 10 per cent of each other) repeat, using a 1:100 or 1:200 dilution of vaccine.

10. Divide the total bacteria in 20 squares by 20 to obtain the average per square.

11. Multiply by 400,000,000 to give the number per c.c. of dilution:

$$\frac{1}{20} \text{ mm.} \times \frac{1}{20} \text{ mm.} \times \frac{1}{50} \text{ mm.} = \frac{1}{20,000} \text{ c.mm. (contents of a square)}$$

$$\frac{1}{20,000} \times \frac{1}{20} \text{ (dilution in pipet)} = \frac{1}{400,000} \text{ c.mm. or } \frac{1}{400,000,000} \text{ c.c.}$$

12. Then multiply by 50 (if an original 1:50 dilution was employed) to obtain the number of organisms per c.c. of undiluted suspension.

13. Instead of the above, the Petroff-Hauser (Fig. 235) bacteria counter gives excellent results:

With a *red corpuscle* pipet, draw undiluted suspension to the mark 0.5 and

stain (same as above) to 101 to give a 1:200 dilution. After thorough agitation, discard a few drops and place a drop into the counting cell. Adjust the cover glass and allow fifteen minutes for thorough settling. Make a count of 10 to 20 squares with the following formula:

$$\frac{\text{total bacteria counted} \times 200 \times 20,000,000}{\text{number of small squares counted}} = \text{bacteria per c.c.}$$

14. Place the counting chambers and cover slides in 2 per cent cresol for at least five minutes before wiping. Clean the pipets in the same before drying.

15. After counting, the vaccine is sterilized and diluted to proper strength as described below.

Wright's Method.—1. Make a mark on the stem of a capillary pipet about one inch from the tip and fit a rubber bulb to its barrel.

2. Cleanse and prick the finger.

3. Draw up into the capillary pipet sodium citrate solution to the mark on the tip. Then draw a little air in, then blood from the finger up to the mark. Draw a little air in to separate the solution, next draw bacterial suspension up to the mark (Fig. 236).

4. Expel the contents of the pipet on a glass slide or in a watch glass and mix thoroughly by aspirating and reëxpelling about a dozen times (Fig. 237).

5. Make two or three thin films on slides in the same manner as described for blood smears (Fig. 238).

6. Dry in air and fix with a saturated solution of corrosive sublimate.

7. Wash and stain with dilute carbolfuchsin (1:10) or carbolthionin for two to five minutes.

8. Wash and dry.
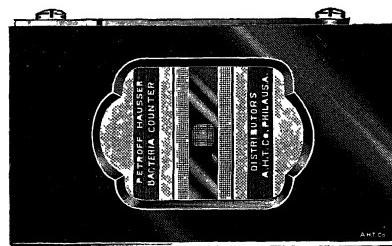
FIG. 235—THE PETROFF-HAUSSER COUNTER.
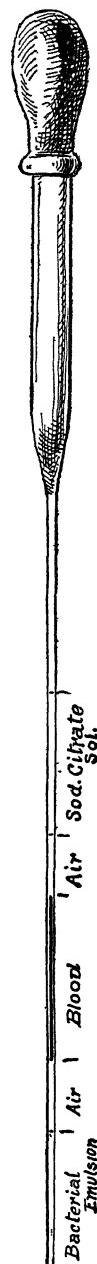
FIG. 236.—A CAPILLARY PIPET FOR COUNTING A BACTERIAL VACCINE

(From Kolmer, Infection, Immunity and Biologic Therapy, W. B. Saunders Co.)

9. Examine only satisfactory films which show bacteria and blood cells in approximately the same numbers and free from bacterial aggregates (Fig.

239). With oil-immersion lens count the number of corpuscles and bacteria in a number of fields, or until 500 corpuscles have been counted. Mark down the number of cells and bacteria counted separately and total each at the end.

10. Calculation: Let us assume that 500 red cells and 1000 bacteria have been counted. One c.mm. of blood contains 5,000,000 red corpuscles and equal vol-
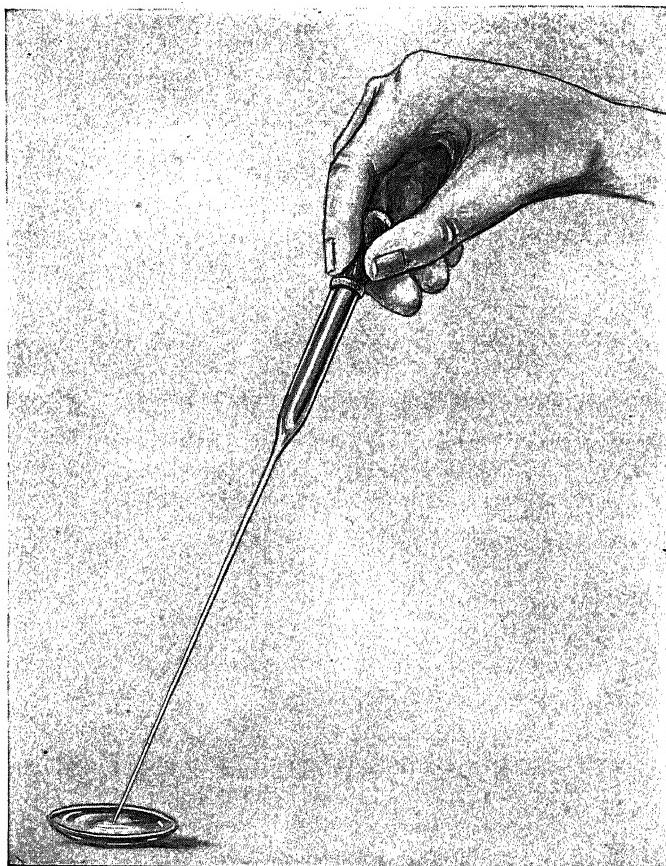
FIG. 237.—MIXING THE CONTENTS OF PIPET.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

umes of blood and emulsion were taken. One c.mm. of the emulsion, therefore, contains $\dfrac{5,000,000 \times 1000}{500} = 10,000,000$ organisms per c.mm., or 10,000,000,000 per c.c.

11. After counting the vaccine is sterilized and diluted to proper strength as described below.

Nephelometer Method (McFarland).—1. *This method is mainly applicable for suspensions prepared from agar slants or from centrifuged broth cultures*

resuspended in saline solution and containing no coloring matter from the medium (see page 402 for preparation with coloring matter).

FIG. 238.—SMEARS FOR VACCINE COUNTS.

(From Kolmer, *Infection, Immunity and Biologic Therapy,* W. B. Saunders Co.)

2. Place 1 c.c. of bacterial suspension in test tube which should be of the same size as those used in the nephelometer (Fig. 240).

3. Dilute with 4 to 10 c.c. of salt solution, keeping accurate record of the final dilution.

4. Compare with tubes of nephelometer. Shake the tubes well before comparing.

5. Calculation: Multiply the number of bacteria represented by the nephelometer tube which corresponds with the density of the bacterial suspension by the dilution of the bacterial suspension. For example, assume that the density of the bacterial suspension corresponds to the No. 3 tube of the nephelometer and before making comparison it was diluted 8 times. No. 3 tube corresponds to 1,000,000,000; 8 times this number equals 8,000,000,000 bacteria per c.c.

6. The nephelometer is prepared as follows:

FIG. 239.—COUNTING VACCINE.
(From Kolmer, *Infection, Immunity and Biologic Therapy,* W. B. Saunders Co.)

(*a*) Arrange ten test tubes or ampules of uniform size in rack, and label 1 to 10.

(*b*) Add the following amounts of a 1 per cent solution of chemically pure

barium chloride: To tube No. 1, 0.1 c.c.; tube No. 2, 0.2 c.c.; and so on, increasing 0 1 c c. in each.

(c) Add sufficient of a 1 per cent chemically pure sulphuric acid solution to make the total volume in each tube 10 c.c.

(d) Seal the tubes or ampules.

(e) When the fine white precipitate of barium sulphate which has formed in the tubes is shaken up well, each tube will have a different density, increas-
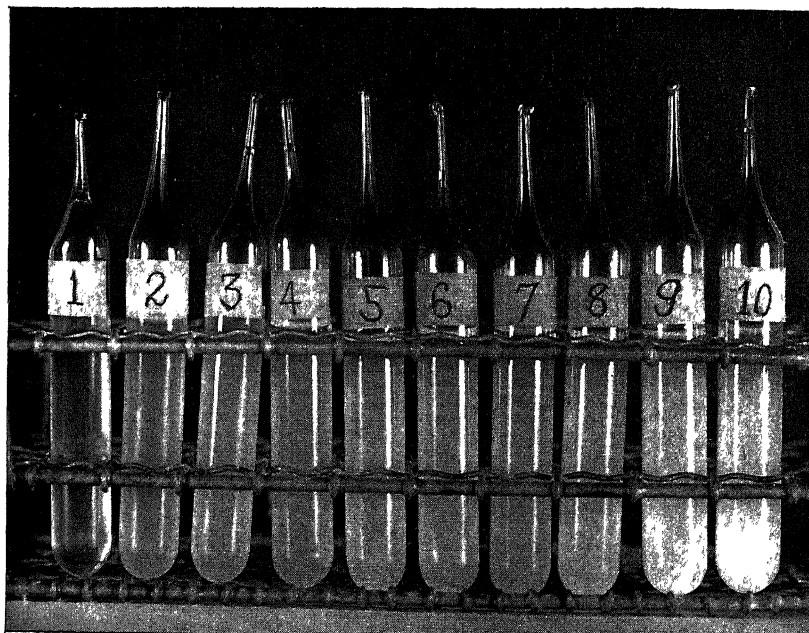
FIG 240 —NEPHELOMETER.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co)

ing from Nos. 1 to 10. The density of the tubes corresponds *approximately* to bacterial suspensions, as follows:

No 1.	300,000,000		No 6:	1,800,000,000
No 2:	600,000,000		No 7.	2,100,000,000
No 3:	900,000,000		No. 8.	2,400,000,000
No. 4:	1,200,000,000		No. 9:	2,700,000,000
No 5:	1,500,000,000		No 10:	3,000,000,000

7. If vaccines are prepared of broth cultures, the nephelometer should be prepared with 1 per cent sulphuric acid in broth in order to convey the color of the latter.

DILUTING, STERILIZING AND PRESERVING VACCINES

Chemical Sterilization.—1. Vaccines sterilized with tricresol, cresol or phenol without the aid of heat are commonly regarded as being more antigenic than heat-killed vaccines.

2. Tricresol is recommended in a final concentration of 0.5 per cent for sterilization and preservation A 5 per cent stock solution in water may be employed, adding 1.0 c.c. for each 10 c.c. of vaccine to give the final concentration of 0 5 per cent.

3. Proceed as per the following example: 20 c.c. of vaccine containing 1,000,000,000 per c.c. is desired. The suspension contains 2,700,000,000 per c.c.

$$\frac{1000 \times 20}{2700} = 7.0 \text{ c.c. of suspension to be used}$$

4. In a sterile vial or bottle containing a few glass beads place: 7.0 c c. of suspension; 2.0 of 5 per cent tricresol; 11 c c. of sterile saline.

5. If the vaccine is to be a mixed one, prepare separate vaccines of each organism in this manner and then mix equal parts of each.

6. Stopper with a sterile rubber cap, mix and place in the incubator at 37° C. for twenty-four hours.

7. Remove 0.5 c.c. with a sterile syringe and needle and place in a tube of at least 10 c.c. of a suitable broth medium for sterility test. Incubate twenty-four to forty-eight hours and dispense the vaccine as ready for administration if sterile.

8. If not sterile, reculture the vaccine. As a general rule, twenty-four hours at 37° C. are sufficient unless spores are present.

Sterilization by Heat.—1. Stopper the vial or bottle with a rubber cap and immerse in a bath of cold water reaching above the level of the vaccine.

2. Place a thermometer in the bath, bring the temperature up to 60° C. and hold it for one hour.

3. Make a culture of the vaccine for sterility as described above.

4. If not sterile, the vaccine may be reheated for another hour although this may reduce its antigenic activity. As a general rule, one hour at 60° C. is sufficient unless contaminating spores are present, in which case it should be discarded.

METHODS FOR DISPENSING VACCINES

1. It is quite convenient to dispense vaccines in vials or small bottles (Fig. 241) stoppered with rubber caps.

2. The first dose may be 0.1 c.c. and subsequent doses gradually increased, as by 0 1 or 0.2 c.c., according to reactions. These amounts are readily removed with a suitable syringe and needle after disinfecting the rubber cap with tincture of iodine or some other suitable disinfectant.

3. Some physicians prefer having vaccines dispensed in ampules (Fig. 242).

4. The designated doses, as 0 1, 0.2, 0 3, 0 4 c c, etc, are placed in small sterile ampules with a sterile pipet and the volume in each brought up to 1 c c. by adding tricresolized saline solution (0 3 c.c. of tricresol in 100 c.c. of saline).

FIG 241—BOTTLE AND CAP FOR
BACTERIAL VACCINE
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W B Saunders Co)
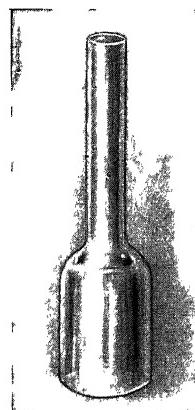
FIG 242—A SMALL
VACCINE AMPULE
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W B Saunders Co)

The neck of each ampule is then sealed in a flame and each labeled with a number or the dose.

PREPARATION OF FILTRATE VACCINE

1. This method is particularly suitable for preparing vaccines of staphylococcus toxins (filtrates).

2. Cultivate *Staphylococcus aureus* (hemolytic) in broth at 37° C. for four to five days.

3. Filter through a small sterile Berkefeld or Mandler filter.

4. Test the filtrate for sterility by culturing 0 5 c.c. in a tube of broth for at least twenty-four hours.

5. Do not heat, as this destroys staphylococcus toxin.

6. For preservative, add 0.6 c c. of 5 per cent tricresol to each 10 c c of filtrate or the filtrate may be used without a preservative if due precautions are taken against contamination

7. The first dose for an adult should not exceed 0.05 c.c., as these vaccines produce more local and systemic reactions than ordinary heat-killed vaccines.

8. If necessary, the filtrate may be diluted with tricresolized saline (0 3 c.c. tricresol in 100 c.c of saline solution).

PREPARATION OF AUTOGENOUS VACCINES

Kolmer's Method.—1 This method is based upon employing any exogenous toxins produced by bacteria as well as the organisms themselves.

2. A pure culture of the organism is cultivated at 37° C for at least four to five days in a hormone broth medium (do not use serum or blood broth). If there are two or more organisms, each is grown separately in pure culture and made up into separate vaccines, which are finally mixed together in equal proportions to give a single vaccine of desired numerical strength.

3. The broth culture is counted by the counting chamber or Wright method described above or estimated by a nephelometer prepared with broth (page 402).

4. Proceed as per following example: 20 c c. of vaccine containing 1,000,000,000 per c c. are desired. Count shows 5,200,000,000 per c.c.

$$\frac{1000 \times 20}{5200} = 3\,6 \text{ c c. of suspension to use}$$

5. In a sterile vial or bottle containing a few glass beads place: 3.6 c.c. of broth suspension; 20 c c. of 5 per cent tricresol; 14 4 c c of sterile saline.

6. If the vaccine is to be a mixed one prepare separate vaccines of each organism in this manner and then mix equal parts of each

7. Stopper with a sterile rubber cap, mix and incubate at 37° C. for twenty-four hours. Do not heat at 55° to 60° C. as this destroys thermolabile toxins and particularly those produced by staphylococci.

8. Culture 0 2 c c. in at least 10 c c of a suitable broth medium for sterility.

9. If sterile, the vaccine is ready for administration.

10 Vaccines prepared by this method are of a light brown color and may give slightly more local reaction at the site of infection. The first dose may be 0.1 or 0.2 c.c and subsequent doses slightly increased

Cohen's Pathogen-Selective Method.—1. This method aims to prepare autogenous vaccines mainly of organisms found capable of growing in the whole, coagulable blood of the patient on the assumption that these are most pathogenic for the individual.

2. Secure material to be cultured on sterile swabs (from nose, tonsils, extracted teeth, tooth sockets, sputum, pus, etc), and rub each swab on the

bottom of dry sterile tubes into which one or two drops of broth have been placed.

3. Then place the swabs into tubes of hormone broth medium for controls and also for use in preparing vaccine.

4. Immediately secure from 10 to 12 c.c. of blood from a vein at the elbow of the patient with a sterile syringe and with aseptic precautions.

5. Place 3 to 5 c.c. of blood in each tube in which material has been smeared.

6. Incubate these blood and the control tubes for twenty-four hours. Examine each by smear. If necessary, plate each on blood agar for identification of organisms. Also inoculate a tube of hormone broth from each blood tube showing a growth as a check on the plate and for preparing vaccine

7. The vaccine is now made up by mixing 1 part of the original broth culture (prepared in step 2 above) and 9 parts of the broth subculture of the blood.

8. Count the mixed suspension and finish up the vaccine according to steps 3, 4, 5 and 6 of the Kolmer method described above.

PREPARATION OF TYPHOID-PARATYPHOID VACCINE

1. Use the Rawlings strain of *B. typhosus*; the Kessel strain of *B. paratyphosus A* and the Rowland strain of *B. paratyphosus B*. These may be obtained from the Army Medical School, Washington, D. C.

2. Culture each strain in a tube of broth and examine for purity.

3. Inoculate Blake bottles of agar with each strain if a large amount of vaccine is to be prepared; for smaller amounts inoculate 24 slants of agar with *B. typhosus,* 24 with *B. paratyphosus A* and 24 with *B. paratyphosus B.*

4. Incubate for two to three days and examine for purity.

5. Prepare separate *heavy* suspensions of the three organisms by washing off the agar cultures with appropriate amounts of sterile saline solution.

6. Shake each suspension with sterile glass beads to break up clumps and filter each through sterile paper.

7. Count each suspension by the counting chamber method described above.

8. The finished vaccine should contain in each c.c.:

<div style="text-align:center">

1,000,000,000 *B. typhosus*

750,000,000 *B. paratyphosus A*

750,000,000 *B. paratyphosus B*

</div>

9. Proceed as per the following example: It is desired to make 500 c.c. of finished triple vaccine. Count of typhoid suspension is 8,200,000,000 per c.c. Count of para A suspension is 6,500,000,000 per c.c. Count of para B suspension is 7,900,000,000 per c.c.

$$\frac{1000 \times 500}{8200} = 61 \text{ c c. of typhoid suspension to be used}$$

$$\frac{750 \times 500}{6500} = 57.7 \text{ c.c. of para A suspension to be used}$$

$$\frac{750 \times 500}{7900} = 47.5 \text{ c.c. of para B suspension to be used}$$

10. In a sterile bottle place: 61 c.c. of typhoid suspension; 57.7 c.c. of para A suspension; 47.5 c.c. of para B suspension; 75 c.c. of 2 per cent solution of tricresol; 258 8 c.c. of sterile saline solution.

11. Mix well. This gives 500 c.c. of triple vaccine of the desired strength of each organism preserved with 0.3 per cent tricresol.

12. Place the bottle in a bath of cold water reaching above the level of the vaccine.

13. Place a thermometer and heat up to 54° to 55° C. and hold for one hour.

14. Culture 1 c.c. in a small flask of broth for sterility; incubate for forty-eight hours.

15. For adults the doses at weekly intervals are as follows by subcutaneous injection: 0.5, 1.0, 1.0 and 1.0 c.c.

PREPARATION OF BESREDKA'S ANTIVIRUS

1. Cultivate pure cultures on agar or blood agar slants.

2. Inoculate 1 per cent glucose broth and cultivate at 37° C. for four days.

3. Filter through a sterile Berkefeld or Mandler filter.

4. Reinoculate the filtrate with the same organism carried along on the agar or blood agar slants.

5. Cultivate for four days.

6. Reinoculate a third time and cultivate for four days.

7. Filter again through a sterile Berkefeld or Mandler filter.

8. The filtrate constitutes the "antivirus" and is used in treatment by local application.

9. Or the "antivirus" may be prepared without filtration as follows: (*a*) Inoculate glucose broth and cultivate for four days. (*b*) Reinoculate and cultivate for an additional four days. (*c*) Reinoculate a third time and cultivate for four days. (*d*) Standardize by counting or with a nephelometer; heat at 60° C. for 1 hour; test for sterility; add tricresol as a preservative as described on page 403.

CHAPTER XXI

METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF MILK

Principles.—1. The methods here given are the standard methods of milk analysis of the American Public Health Association and the Association of Official Agricultural Chemists. They are given herewith for guiding the examination of milk in clinical laboratories, especially those connected with hospitals.

2. The total bacterial count continues to be of most value in the bacteriological examination of milk and is especially useful as a measure of the care with which milk is collected and kept until used.

3. Methods for the detection of tubercle bacilli in milk are described on page 361. Unfortunately, however, there are no practical methods at present for the detection of typhoid and dysentery bacilli, *B abortus*, scarlet fever streptococci, and other pathogenic organisms known to be sometimes transmitted by milk. It is still necessary to rely mainly for the elimination of these upon thorough pasteurization, veterinary inspection of herds, and the medical examination and supervision of dairy employees with special reference to typhoid carriers.

4. It is true, however, that the presence of mastitis in cows is sometimes to be detected by finding exceedingly large numbers of long-chained streptococci and pus cells when due care is taken to examine the milk within six hours after collection or when carefully refrigerated to prevent multiplication. Due care, however, must be exercised in the examination of sediments secured by centrifuging against mistaking normal streptococci and those contained in butter starters or derived from dirty milking machine tubes, for pathogenic streptococci.

STANDARD PLATE METHOD FOR TOTAL BACTERIAL COUNTS

1. If bottled milk is to be examined, the bottle should be immediately iced. In case the milk is in bulk, a sample should be taken after thorough mixing. A sterile glass tube long enough to reach from the top to the bottom of the container to be sampled is very satisfactory. The tube is lowered to the bottom and the end closed with the thumb or a finger to hold the contents in the tube, which is then placed in a sample bottle. The sample bottle should be sterile and large enough to hold the entire amount in the tube. Sample bottles

should be glass stoppered as cotton plugs are not satisfactory. *Do not collect less than 10 c.c.*

2. If the sample is not to be examined immediately, place it in cracked ice so as to cool promptly to near the freezing point. Prepare dilution bottles to contain 99 c.c. of water after sterilization in the autoclave. They should have rubber or glass stoppers. The number of bottles will depend upon the number of samples to be examined and dilutions desired.

3. Shake sample twenty-five times, each shake being an up and down excursion of about one foot. Then immediately transfer 1 c.c. to dilution bottle No. 1 (this makes a dilution of 1 : 100).

4. Shake dilution No. 1 twenty-five times and transfer 1 c.c. to dilution bottle No. 2. At the same time transfer 1 c.c. and 0.1 c.c. to two empty sterile Petri dishes. Mark the plates 1 : 100 and 1 : 1000.

5. Shake dilution No. 2 and transfer 1 c.c. and 0.1 c.c. to two empty Petri dishes and mark them 1 : 10,000 and 1 : 100,000. A special pipet is recommended which delivers 1.1 c.c.

6. Melt nutrient beef extract agar with a P^H of 6.6 and cool to between 40° and 45° C.

7. Pour 10 c.c. into each plate and mix with the diluted milk by gently rotating. Sufficient agar should be used to avoid drying out. An excess will favor the spreading of surface colonies.

8. Incubate all plates for forty-eight hours at 37° C. in an inverted position.

9. Select plates showing between 30 and 300 colonies. Count the number of colonies on the plates, using a lens magnifying about 2.5 diameters. A

FIG 243 —STEWART COUNTING APPARATUS.

colony counting chamber with uniform illumination and with standard ruling should be used (Fig. 243). If the number of colonies exceeds 300, a fraction of the plate can be counted and the number multiplied by the factor and then by the dilution. Plates with less than 20 colonies should not be counted unless no others are available. When many samples are counted daily, Robinson's electric counting device may be found useful and time-saving (Fig. 244). In case of doubt, the compound microscope may be used to distinguish between colonies and débris.

10. Multiply the numbers of colonies by the dilution marked on the plate. This will give the number of bacteria per c.c. of milk. Use only two significant left-hand digits in any report. Raise to the next highest round number but never lower.

11. *A series of at least four or more samples should be examined before judging the quality of a given milk supply.*

FIG 244—ROBINSON'S COLONY COUNTER.

12. In addition to other requirements, the bacterial counts required before delivery of various grades of milk according to the standard milk ordinance of the United States Public Health Service, are as follows:

Certified Milk: Less than 10,000 per c c.
Grade "A" Raw: Not over 50,000 per c.c.
Grade "B" Raw: Not over 200,000 per c.c.
Grade "C" Raw: Not over 1,000,000 per c.c.
Grade "D" Raw: Not over 5,000,000 per c c.
Grade "A" Pasteurized: Not over 10,000 per c c.
Grade "B" Pasteurized: Not over 100,000 per c c.
Grade "C" Pasteurized: Not over 500,000 per c c.

DIRECT MICROSCOPIC COUNT OF BACTERIA

Breed Method.—1. The collection of sample is the same as described above for the plate count.

2. Thoroughly shake the sample and deposit 0.01 c.c. of the milk on a clean slide by means of a special pipet (Fig. 245).

FIG 245—BREED AND BREW CAPILLARY PIPET

3. Spread the milk evenly over an area of 1 centimeter with a clean stiff needle. The slide can be laid on paper ruled in 1 centimeter squares or on any ruled guide plate (Fig. 246). This ruling will show the area to be covered by the smear.

4. Dry the film in a warm place. Avoid excess heat as it may cause the film to crack. The drying should be complete within five to ten minutes.

5. Dip the slide in xylol to remove the fat (at least one minute). Drain and allow to dry.

6. Place in 90 per cent alcohol for one or more minutes.

7. Transfer to Loffler's methylene blue for five minutes (to overstain).

8. Rinse in water and then decolorize with alcohol. Check the decolorization by observation to avoid overdecolorizing. When properly done the background should show a faint pale blue. If decolorization is carried too far, the smear can be re-stained.

FIG 246 —BREED AND BREW GUIDE PLATE

9. Or the following stain, devised by Newman, may be employed:

Methylene blue (certified powder)	1 12 gm.
Ethyl alcohol (95 per cent)	54 00 c c.
Tetrachlorethane (tech.)[1]	40.00 c.c.
Glacial acetic acid .. .	6.00 c c

Add the alcohol to the tetrachlorethane in a flask and bring to a temperature not to exceed 70° C. (If it is desired to use methyl alcohol the temperature should not be raised to more than 55° to 60° C.) Add the warm mixture to the powdered methylene blue. Shake vigorously until the dye is completely dissolved, then add slowly the glacial acetic acid to the cold solution. Agitate the flask during addition of acid. Filter the entire volume through a 15 centimeter filter paper. Keep in tightly stoppered bottle. Allow the stain to act for thirty seconds. Steps 5 and 6 of the above may be omitted as the fat is removed by the stain.

10. Adjust the microscope so the field of vision is 0.205 millimeter in diameter. This can be done by using a stage micrometer with a 1.9 millimeter (oil-immersion) objective and a 6.4× ocular; adjust the tube until the field has the required size.

Count the number of bacteria seen in 30 fields. Each field represents one three hundred thousandth part of a c.c. of the milk.

$$\frac{\text{number of bacteria}}{30} \times 300,000 = \text{number of bacteria per c.c.}$$

or

$$\text{number of bacteria} \times 10,000$$

11. For some purposes, especially when examining low count milk, it is advisable to use a special ocular micrometer with circular ruling divided into

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[1] Obtained from Eastman Kodak Co.

quadrants (Fig. 247). Adjust the microscope so the diameter of the circle is 0.146 millimeter. Count 60 fields and multiply the number of bacteria by 10,000.

12. This direct microscopic method is excellent for making a rapid survey of either raw or pasteurized milk and should be more frequently used as a check on counts obtained by the plate method.

FIG. 247—OCULAR MICROMETER DISK OF BREED AND BREW.

METHYLENE BLUE REDUCTION METHOD

This test is also known as the reductase test. It is based on the principle that the color imparted to milk by a small quantity of methylene blue will disappear more or less quickly. The rate of this decolorization depends largely upon the reducing activity of bacteria. This in turn has been empirically correlated with the number of bacteria when the test is conducted under the usual circumstances and if other factors such as the temperature of the milk are kept constant during the test.

1. Collect samples with same care as for other types of bacteriological examinations.

2. Place 10 c.c. of the milk in a sterile test tube.

3. Add 1 c.c. of methylene blue solution made by adding 1 tablet to 200 c.c. of water (standard methylene blue tablets are prepared by the National Aniline Company and can be obtained from the usual supply houses).

4. Mix the dye thoroughly. This can be done by blowing through the pipet used to add the dye. The milk should now have a robin's-egg blue color.

5. Place the tubes in a water bath at 37° C.

6. Observe frequently. The end-point is to be taken as the time when the blue color has disappeared and the milk has regained its normal color. In the majority of cases the color disappears uniformly throughout the entire mass of milk. With certain samples the color may persist at the surface, or again it may persist at the bottom of the tube. In case the color disappears in an uneven manner, the end-point can be taken as the time when the milk after mixing shows no blue color.

7. The results are interpreted as follows:

Class 1. Good milk: not decolorized in five and a half hours; developing as a rule, less than 500,000 colonies per c.c. on agar plates.

Class 2. Milk of fair quality: decolorized in less than five and a half hours but not less than two hours; developing as a rule, 500,000 to 4,000,000 colonies per c.c. on agar plates.

Class 3. Bad milk: decolorized in less than two hours, but not less than twenty minutes; developing as a rule, 4,000,000 to 20,000,000 colonies per c.c. on agar plates.

Class 4. Very bad milk: decolorized in twenty minutes or less; developing as a rule, over 20,000,000 colonies per c.c. on agar plates.
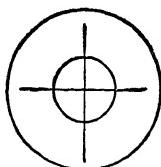
CHAPTER XXII

METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF WATER

Principles.—1. The methods here given are the standard methods for the examination of water adopted by the American Public Health and American Water Works Associations.

2. From the standpoint of the clinical laboratory, examinations for bacilli of the coli-aerogenes group of fecal origin are of most value in relation to the spread of typhoid fever, cholera, and dysentery.

3. Direct examinations for typhoid and paratyphoid bacilli have been previously referred to but are of so very little value that most reliance is placed upon finding colon bacilli as indicative of possible contamination with human fecal material.

COLLECTION

1. Samples for bacterial analysis shall be collected in bottles which have been cleansed with care, rinsed in clean water, and sterilized.

2. Great care must be exercised to have the samples representative of the water to be tested and to see that no contamination occurs at the time of filling the bottles or prior to examination.

3. Because of the rapid and often extensive changes which may take place in the bacterial flora of bottled samples when stored even at temperatures as low as 10° C., it is urged, as of importance, that all samples be examined as promptly as possible after collection.

4. The time allowed for storage or transportation of a bacterial sample between the filling of the sample bottle and the beginning of the analysis should be *not more than six hours for impure waters* and *not more than twelve hours for relatively pure waters.* During the period of storage, the temperature shall be kept between 6° C. and 10° C. Any deviation from the above limits shall be so stated in making reports.

TOTAL BACTERIAL COUNTS

1. Have ready the following: (*a*) Nutrient extract agar or nutrient gelatin with P^H of 6.2 to 7.0 liquefied and cooled to 40° C. (*b*) Dilution bottles containing 9 c.c. or 99 c.c. of water and sterilized at 120° C. for fifteen minutes.

2. When dilutions are made the sample bottle should be shaken vigorously twenty-five times and 1 c.c. withdrawn and added to the proper dilution bottle

as required.   Each dilution bottle shall be shaken vigorously twenty-five times before a second dilution is made from it or before a sample is removed for plating.

3 Plating should be done immediately after making dilutions.   After vigorous shaking twenty-five times, 1 c c. of the sample or dilution shall be placed in the Petri dish.   Ten c c. of liquefied medium (agar or gelatin) at a temperature of 40° C. shall be added to the Petri dish.   The cover of the Petri dish shall be lifted just enough for the introduction of the pipet or culture medium, and the lips of all test tubes or flasks used for pouring the medium shall be flamed.   The medium and sample in the Petri dish shall be thoroughly mixed and uniformly spread over the bottom of the Petri dish by tilting and rotating the dish.   All plates shall be solidified as rapidly as possible after pouring and placed immediately in the incubator.

4. *Gelatin plates* shall be incubated for forty-eight hours at 20° C in a dark, well-ventilated incubator in an atmosphere practically saturated with moisture.

Agar plates may be used for counts made either at 20° C. or 37° C.   The time for incubation at 20° C. shall be forty-eight hours and at 37° C. twenty-four hours.   The incubator shall be dark, well-ventilated and the atmosphere shall be practically saturated with moisture   Glass-covered plates shall be inverted in the incubator.   Any deviation from the above described method shall be stated in making reports.

In making report of the water examination the medium used for the total count should be stated, *i.e*, whether gelatin or agar, and the temperature of incubation given.

5 In preparing plates, such amounts of the water under examination shall be planted as will give from 30 to 300 colonies on a plate ; and the aim should be to always have at least two plates giving colonies between these limits. Where it is possible to obtain plates showing colonies within these limits, only such plates should be considered in recording results, except where the same amount of water has been planted in two or more plates, of which one gives colonies within these limits, while the others give less than 30 or more than 300. In such case, the result recorded should be the average of all the plates planted with this amount of water.   Ordinarily it is not desirable to plant more than 1 c c of water in a plate; therefore, when the total number of colonies developing from 1 c.c. is less than 30, it is obviously necessary to record the results as observed, disregarding the general rule given above.

Counting shall in all cases be done with a lens of 2 5 diameters' magnification, with a focal distance of 3½ inches.   The Engraver's Lens No. 146 made by the Bausch and Lomb Optical Company fills the requirements and is a convenient lens for the purpose.

6. In order to avoid fictitious accuracy and yet to express the numerical results by a method consistent with the precision of the work, the numbers of colonies of bacteria per c.c. shall be recorded as follows:

From 1 to 50 shall be recorded as found.

From 51 to 100 shall be recorded to the nearest 5.

From 101 to 250 shall be recorded to the nearest 10.

From 251 to 500 shall be recorded to the nearest 25

From 501 to 1000 shall be recorded to the nearest 50.

From 1001 to 10,000 shall be recorded to the nearest 100.

From 10,001 to 50,000 shall be recorded to the nearest 500.

From 50,001 to 100,000 shall be recorded to the nearest 1000.

From 100,001 to 500,000 shall be recorded to the nearest 10,000

From 500,001 to 1,000,000 shall be recorded to the nearest 50,000.

From 1,000,001 to 10,000,000 shall be recorded to the nearest 100,000.

This applies to the gelatin count at 20° C. and to the agar counts at 20° C. and 37° C.

DETERMINATION OF THE PRESENCE OF MEMBERS OF THE COLI-AEROGENES GROUP

The coli-aerogenes group is to be considered as including all gram-negative non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media.

The test described under this heading is really a combination of three tests. The first is called the "presumptive test" and is conducted in all cases. The second is called the "partially confirmed test" and is used to confirm the first test when doubtful. The third is the "completed test," used when the results of the second test are doubtful.

Presumptive Test.—1. Inoculate a series of fermentation tubes containing lactose broth (nutrient broth containing 0 5 per cent lactose) with the following amounts of the water to be tested: 10, 1, 0.1, and 0.01 c.c.

2. The amount of media should always equal at least twice the amount of water inoculated Any type of fermentation tube can be used. The Dunham tube with inverted vial is recommended. When required to examine larger amounts than 10 c.c., as many tubes as necessary shall be inoculated with 10 c.c. each.

3. Incubate at 37° C. for forty-eight hours.

4. Examine each tube at the end of twenty-four and forty-eight hours.

5. The production within twenty-four hours of gas occupying more than 10 per cent of the inverted vial in the fermentation tube constitutes a *positive presumptive test*

6. If no gas is formed in twenty-four hours, or if the gas formed is less than 10 per cent, the incubation shall be continued to forty-eight hours. The presence of gas in any amount in such a tube at forty-eight hours constitutes a *doubtful test,* which in all cases requires confirmation.

7. The absence of gas formation after forty-eight hours incubation constitutes a *negative test,*

Partially Confirmed Test.—1. Streak or spread Endo or eosin-methylene blue plates from the tube which shows gas formation from the smallest amount of water tested. The transfer should be made as soon as possible after gas formation occurs. If gas formation occurs at the end of twenty-four hours, make transfer at that time. If at the end of forty-eight hours gas has formed in tubes containing less of the sample of water than at twenty-four hours, transfers should be made from these tubes.

2. Incubate at 37° C. for eighteen to twenty-four hours.

3. The results are interpreted as follows: (*a*) If typical colonies have developed upon the plate within this period, the confirmed test may be considered positive. (*b*) If, however, no typical colonies have developed within twenty-four hours, the test cannot yet be considered definitely negative, since it not infrequently happens that members of the coli-aerogenes group fail to form typical colonies on Endo or eosin-methylene blue plates, or that the colonies develop slowly. In such case, it is always necessary to complete the test as described below.

Completed Test.—1. (*a*) *From typical plates*: From the Endo or eosin-methylene blue plates showing typical colonies, fish at least two colonies, transferring each to an agar slant and a lactose fermentation tube.

(*b*) *From atypical plates:* If no typical colonies appear upon the plate within twenty-four hours, the plate should be incubated another twenty-four hours, after which at least two of the colonies considered most likely to be organisms of the coli-aerogenes group whether typical or not shall be transferred to agar slants and lactose fermentation tubes.

2. Incubate the lactose broth fermentation tubes until gas formation is noted, the incubation not to exceed forty-eight hours. The agar slants shall be incubated at 37° C. for twenty-four hours, when a microscopic examination shall be made of at least one culture, selecting the one which corresponds to one of the lactose broth fermentation tubes which has shown gas formation.

3. The formation of gas in lactose broth and the demonstration of gram-negative non-spore-forming bacilli in the agar culture shall be considered a satisfactorily completed test, demonstrating the presence of a member of the coli-aerogenous group. The absence of gas formation in lactose broth or failure to demonstrate gram-negative non-spore-forming bacilli in a gas-forming culture constitutes a negative test.

Interpretation of the Results.—Presumptive Test.—1. When definitely positive, that is, showing more than 10 per cent of gas in twenty-four hours, this test is sufficient:

(*a*) As applied to all except the smallest gas-forming portion of each sample in all examinations.

(*b*) As applied to the smallest gas-forming portion in the examination of sewage or of water showing relatively high pollution, such that its fitness for use as drinking water does not come into consideration. This applies to the

routine examination of raw water in connection with control of the operation of purification plants.

2. When definitely negative, that is, showing no gas in forty-eight hours, this test is final and therefore sufficient in all cases.

3. When doubtful, that is, showing gas less than 10 per cent (or none) in twenty-four hours, with gas either more or less than 10 per cent in forty-eight hours, this test must always be confirmed.

PARTIALLY CONFIRMED TEST.—1. When definitely positive, that is, showing typical plate colonies within twenty-four hours, this test is sufficient:

(a) When applied to confirm a doubtful presumptive test in cases where the latter, if definitely positive, would have been sufficient.

(b) In the routine examination of water supplies where a sufficient number of prior examinations have established a satisfactory index of the accuracy and significance of this test in terms of the completed test.

2. When doubtful, that is, showing colonies of doubtful or negative appearance in twenty-four hours, this test must always be completed.

COMPLETED TEST —The completed test is required as applied to the smallest gas-forming portion of each sample in all cases other than those noted as exceptions under the "presumptive" and the "partially confirmed" tests.

The completed test is required in *all* cases where the result of the partially confirmed test has been doubtful.

NOTES.—1. In reporting a single test, it is preferable merely to record results as observed, indicating the amounts tested and the result in each, rather than to attempt expression of the result in number of organisms per c.c. In summarizing the results of a series of tests, however, it is desirable, for the sake of simplicity, to express the results in terms of the number of coli-aerogenes organisms per c.c., or per 100 c.c. The number per c.c. is the reciprocal of the smallest portion (expressed in c.c.) giving a positive result. For example, the result 1 c.c. plus, 0.1 c.c. plus, 0.01 c.c. negative, would be recorded as 10 per c.c. An exception should be made in the case where a negative result is obtained in an amount larger than the smallest portion giving a positive result; for example, in a result such as 10 c.c. plus, 1 c c. minus, 0.1 c.c. plus. In such case, the result should be recorded as indicating a number of coli-aerogenes organisms per c.c. equal to the reciprocal of the portion next larger than the smallest one giving a positive test, this being a more probable result.

Where tests are made in amounts larger than 1 c.c., giving average results less than 1 per c.c., it is more convenient to express results per 100 c.c.

2. Recent work seems to indicate that the coli-aerogenes group as herein defined consists of organisms of both fecal and nonfecal origin. Methods for making this distinction are the methyl red, Voges-Proskauer, uric acid, and sodium citrate tests, but none have been as yet adopted as standard. This statement should not be construed as detracting from the value of the group test as above described for the routine examination of water supplies.

CHAPTER XXIII

METHODS FOR TESTING DISINFECTANTS

A very large number of methods have been proposed for testing the bactericidal and bacteriostatic (antiseptic) properties of disinfectants and the method of testing has a tremendous influence upon the results. For this reason one laboratory may report a substance as possessing a high disinfectant value and another that it is practically inert. It is easily possible, therefore, to influence greatly the value placed upon a disinfectant by the method of testing. It is hoped that the methods here given will prove serviceable for the purposes of the clinical laboratory.

HYGIENIC LABORATORY METHOD

No single method can serve as a means of comparing the value in practice of disinfectants of greatly diverse composition and destined for a variety of applications. However, disinfectants which are chemically related to phenol, which are to be used against organisms reacting similarly to the manner in which the typhoid bacillus reacts and which are destructive within the time and temperature limits of this test, may be compared as to their disinfecting properties within these limitations by means of this test. The results may be useful in the selection of a potent product, in making comparisons of cost in terms of service rendered, and in checking successive batches of the same product.

Test Culture.—The test culture is a culture of *B. typhosus*, Hopkins strain. Between periods of testing it is maintained on nutrient agar stabs, transferred at monthly intervals.

For at least five days before the test the culture is transferred at twenty-four-hour intervals to successive tubes of the meat extract broth described below, and incubated at 37° C. Transfers are made with one standard loopful. The culture is filtered through sterile filter paper just before using. The test is performed with a twenty-four-hour culture.

Phenol.—The phenol must comply with the requirements of the *Tenth United States Pharmacopoeia*. Particularly the congealing point must not be below 40° C. The crystals are kept in tightly stoppered amber-colored bottles in a dark and relatively cool place.

A 5 per cent original solution is made by adding 1 part by weight of phenol,

liquefied by warming the bottle, to 19 parts of distilled water. A fresh solution is made for each day's use.

Culture Medium.—Make meat extract medium as follows:

Beef extract (Liebig's)........	3 gm.
Peptone (Armour's for disinfectant testing).	10 gm.
Sodium chloride.............	5 gm.
Water (distilled).......................	1000 c.c.

Boil for fifteen minutes. Make up to original weight by addition of water. Filter through paper. Tube 10 c.c. to each tube. Sterilize. The P^H value of this medium should be between 6.0 and 7.0.

Glassware and Apparatus.—Glassware for measuring must be accurately graduated. It must be clean, dry, and sterile at the time of use. The following will be needed:

  1 c.c. capacity pipets
  5 c.c. capacity pipets
  1 c.c. delivery pipets, graduated in tenths
  5 c.c. delivery pipets
  100 c.c. measuring cylinders, graduated in 1 c.c., glass-stoppered
  Seeding tubes, 1 by 3 inches, flared tops, round bottoms
  Racks consisting of blocks of wood with rows of holes for both the
    seeding tubes (before they are placed in the water bath) and the sub-
    culture tubes

Wire loops must be carefully made and kept from damage. They are made as follows: A close cylindrical spiral is made by winding a piece of platinum wire, No. 23, B. & S. gauge, as tightly as possible about a piece of steel or other hard wire having a diameter of 0.072 inch (No. 13, B. & S. gauge) to complete a little more than four full turns. The long end of the wire is then bent sharply at right angles to the wound portion and parallel to the steel wire. The core is removed and the short end of the wire is clipped off so as to leave exactly four full turns to the coil. The successive turns of the spiral must touch one another continuously. The long end of the wire is attached to an aluminum handle.

A convenient support is provided on which to rest the loops so that a batwing Bunsen burner may be placed under each one successively.

A constant temperature bath is provided, capable of maintaining the seeding tubes at 20° C. during the time of the test. A well-insulated bath of large volume relative to the surface exposed is sufficient without thermoregulating appliances.

DISINFECTANT TESTING MACHINE.—The use of a disinfectant testing machine is optional. One is described in Reprint No. 462 from the *Public Health Reports*. A few modifications have proved useful. For example, the use of platinum instead of nichrome loops, and the practice of sterilizing the subculture tubes covered with padded inverted troughs in the racks.

Dilutions.—Dilutions of phenol and of disinfectants are made from the original liquid on the day of the test. For the dilutions of the disinfectant, a 5 per cent solution is made by adding 5 c.c. of the disinfectant to 95 c.c. of sterile distilled water. A standardized 5 c.c. capacity pipet is used for this purpose. After filling the pipet, all excess of the disinfectant on the outside of the pipet is wiped off with sterile gauze. The contents of the pipet are then delivered into a cylinder containing 95 c.c. of sterile distilled water and the pipet is washed out as clean as possible by aspiration and blowing out the contents into the cylinder. The contents of the cylinder are then thoroughly shaken and the dilutions up to 1 : 500 are made from it, using delivery pipets for measuring. For those disinfectants which do not readily form a 5 per cent solution, make a 1 per cent solution, and from this make the dilutions greater than 1 : 100 in accordance with the second table of dilutions. If greater dilutions than 1 : 500 are to be made, a 1 per cent solution is made from the 5 per cent solution and the higher dilutions are made from this.

For the higher dilutions, delivery pipets may be used. The following scale is used for making dilutions:

For dilutions up to 1 : 70, increase or decrease by a difference of 5 (*i.e.*, 5 parts of water); from 1 : 70 to 1 : 160, by a difference of 10; from 1 : 160 to 1 : 200, by a difference of 20; from 1 : 200 to 1 : 400, by a difference of 25; from 1 : 400 to 1 : 900, by a difference of 50; from 1 : 900 to 1 : 1800, by a difference of 100; from 1 : 1800 to 1 : 3200, by a difference of 200; and so on if higher dilutions are necessary.

It is important that the cylinders used for making the dilutions be correctly graduated. It is preferable to use standardized cylinders and pipets. For making the dilutions in accordance with the above scheme, the accompanying tables are of service.

Method.—This description applies to the hand method. For the use of the machine, follow the procedure described in Reprint No. 462 from the *Public Health Reports*.

1. The object is to add 0.1 c.c. of typhoid culture to 5 c.c. of successive dilutions of the disinfectant and of phenol, and, after this addition, to transfer a loopful of each mixture to a separate subculture tube at periods of five, seven and one-half, ten, twelve and one-half, and fifteen minutes. The subculture tubes are then incubated for forty-eight hours at 37° C. and readings of growth or no growth are made and recorded.

2. Dilutions are made to cover the expected range of the disinfectant, and 5 c.c. of each dilution are placed in a seeding tube. Dilutions of phenol are made as follows: 1 : 80, 1 : 90, 1 : 100, 1 : 110, 1 : 120, and 1 : 130, and 5 c.c. of each are placed in a seeding tube.

3. The seeding tubes are placed in the water bath at 20° C. and a few minutes are allowed for their contents to reach this temperature.

4. To each seeding tube 0.1 c.c. of culture is then added seriatim, allowing fifteen seconds for each addition. If there are ten tubes of disinfected dilu-

5 c.c. of disinfectant + 95 c c. of distilled water = Solution A

Dilution		Solution A, c c		Distilled Water. c c.		Solution A, c c.		Distilled Water, c c.		Solution A, c c.		Distilled Water, c c.
I : 20	=	20	+	0	or	10	+	0	or	4	+	0
I : 25	=	20	+	5	or	10	+	2 5	or	4	+	1
I : 30	=	20	+	10	or	10	+	5	or	4	+	2
I : 35	=	20	+	15	or	10	+	7 5	or	4	+	3
I : 40	=	20	+	20	or	10	+	10	or	4	+	4
I : 45	=	20	+	25	or	10	+	12 5	or	4	+	5
I : 50	=	20	+	30	or	10	+	15	or	4	+	6
I : 55	=	20	+	35	or	10	+	17 5	or	4	+	7
I : 60	=	20	+	40	or	10	+	20	or	4	+	8
I : 65	=	20	+	45	or	10	+	22 5	or	4	+	9
I : 70	=	20	+	50	or	10	+	25	or	4	+	10
I : 70	=	20	+	50	or	10	+	25	or	4	+	10
I : 80	=	20	+	60	or	10	+	30	or	4	+	12
I : 90	=	20	+	70	or	10	+	35	or	4	+	14
I : 100	=	20	+	80	or	10	+	40	or	4	+	16
I : 110	=	20	+	90	or	10	+	45	or	4	+	18
I : 120	=	20	+	100	or	10	+	50	or	4	+	20
I : 130	=	20	+	110	or	10	+	55	or	4	+	22
I : 140	=	20	+	120	or	10	+	60	or	4	+	24
I : 150	=	20	+	130	or	10	+	65	or	4	+	26
I : 160	=	20	+	140	or	10	+	70	or	4	+	28
I : 170	=	20	+	150	or	10	+	75	or	4	+	30
I : 180	=	20	+	160	or	10	+	80	or	4	+	32
I : 200	=	20	+	180	or	10	+	90	or	4	+	36
I : 200	=	20	+	180	or	4	+	36	or	2	+	18
I : 225	=	20	+	205	or	4	+	41	or	2	+	20.5
I : 250	=	20	+	230	or	4	+	46	or	2	+	23
I : 275	=	20	+	255	or	4	+	51	or	2	+	25 5
I : 300	=	20	+	280	or	4	+	56	or	2	+	28
I : 325	=	20	+	305	or	4	+	61	or	2	+	30 5
I : 350	=	20	+	330	or	4	+	66	or	2	+	33
I : 375	=	20	+	355	or	4	+	71	or	2	+	35 5
I : 400	=	20	+	380	or	4	+	76	or	2	+	38
I : 450	=	20	+	430	or	4	+	86	or	2	+	43
I : 500	=	20	+	480	or	4	+	96	or	2	+	48

tions, this will occupy two and one-half minutes. At the end of five minutes from the time of adding the disinfectant to the first seeding tube, a loopful of the mixture is transferred from this tube to a subculture tube, and this is done from each successive seeding tube at fifteen-second intervals. This procedure is repeated after the lapse of seven and one-half, ten, twelve and one-half and fifteen minutes from the time of the first addition of culture to the seeding tube. Each loop is placed on the support and flamed with the Bunsen burner immediately after use, and the use of several loops permits them to cool before they are needed again. The operator is therefore obliged to make a transfer every fifteen seconds for ten minutes.

5. The culture is best added to the seeding tube by holding the latter in a slanting position and touching the tip of the 1 c.c. pipet to the wetted sur-

1 c.c. of disinfectant + 99 c.c. of distilled water = Solution B

Dilution		Solution B, c.c.		Distilled Water, c.c.		Solution B, c.c.		Distilled Water, c.c.		Solution B, c.c.		Distilled Water, c.c.
1 : 100	=	100	+	0	or	10	+	0				
1 : 110	=	100	+	10	or	10	+	1				
1 : 120	=	100	+	20	or	10	+	2				
1 : 130	=	100	+	30	or	10	+	3				
1 : 140	=	100	+	40	or	10	+	4				
1 : 150	=	100	+	50	or	10	+	5				
1 : 160	=	100	+	60	or	10	+	6				
1 : 180	=	100	+	80	or	10	+	8				
1 : 200	=	100	+	100	or	10	+	10				
1 : 200	=	100	+	100	or	10	+	10	or	4	+	4
1 : 225	=	100	+	125	or	10	+	12 5	or	4	+	5
1 : 250	=	100	+	150	or	10	+	15	or	4	+	6
1 : 275	=	100	+	175	or	10	+	17 5	or	4	+	7
1 : 300	=	100	+	200	or	10	+	20	or	4	+	8
1 : 325	=	100	+	225	or	10	+	22 5	or	4	+	9
1 : 350	=	100	+	250	or	10	+	25	or	4	+	10
1 : 375	=	100	+	275	or	10	+	27 5	or	4	+	11
1 : 400	=	100	+	300	or	10	+	30	or	4	+	12
1 : 400	=	10	+	30	or	4	+	12	or	2	+	6
1 : 450	=	10	+	35	or	4	+	14	or	2	+	7
1 : 500	=	10	+	40	or	4	+	16	or	2	+	8
1 : 550	=	10	+	45	or	4	+	18	or	2	+	9
1 : 600	=	10	+	50	or	4	+	20	or	2	+	10
1 : 650	=	10	+	55	or	4	+	22	or	2	+	11
1 : 700	=	10	+	60	or	4	+	24	or	2	+	12
1 : 750	=	10	+	65	or	4	+	26	or	2	+	13
1 : 800	=	10	+	70	or	4	+	28	or	2	+	14
1 : 850	=	10	+	75	or	4	+	30	or	2	+	15
1 : 900	=	10	+	80	or	4	+	32	or	2	+	16
1 : 900	=	5	+	40	or	4	+	32	or	2	+	16
1 : 1000	=	5	+	45	or	4	+	36	or	2	+	18
1 : 1100	=	5	+	50	or	4	+	40	or	2	+	20
1 : 1200	=	5	+	55	or	4	+	44	or	2	+	22
1 : 1300	=	5	+	60	or	4	+	48	or	2	+	24
1 : 1400	=	5	+	65	or	4	+	52	or	2	+	26
1 : 1500	=	5	+	70	or	4	+	56	or	2	+	28
1 : 1600	=	5	+	75	or	4	+	60	or	2	+	30
1 : 1700	=	5	+	80	or	4	+	64	or	2	+	32
1 : 1800	=	5	+	85	or	4	+	68	or	2	+	34
1 : 1800	=	5	+	85	or	4	+	68	or	2	+	34
1 : 2000	=	5	+	95	or	4	+	76	or	2	+	38
1 : 2200	=	5	+	105	or	4	+	84	or	2	+	42
1 : 2400	=	5	+	115	or	4	+	92	or	2	+	46
1 : 2600	=	5	+	125	or	4	+	100	or	2	+	50
1 : 2800	=	5	+	135	or	4	+	108	or	2	+	54
1 : 3000	=	5	+	145	or	4	+	116	or	2	+	58
1 : 3200	=	5	+	155	or	4	+	124	or	2	+	62

face exposed on its upper side. One-tenth c.c. is run in at the proper time and thorough admixture is assured by gentle shaking.

6. The dilutions of phenol are next treated in the same manner as those of the disinfectant.

7. The tubes are properly labeled and are placed in the incubator for forty-eight hours, at the end of which time readings of growth or no growth are made and entered in a table as plus or minus signs, respectively.

8. In the nature of the test it is unavoidable that discrepancies in the even oblique slant of the plus signs across the chart will occasionally be encountered; but if these are numerous, a faulty technic is indicated and the test should be discarded. The same applies to accidentally contaminated tubes.

9. Determination of Coefficient.—The coefficient is the arithmetic mean of the sum of three ratios, expressed decimally. These ratios are, the denominator of the highest dilution of the disinfectant in whose subculture tube no growth occurs, divided by the corresponding figure for phenol, for the five-, ten-, and fifteen-minute intervals, respectively. For example:

Dilution	Time of Exposure in Minutes				
	5	$7\frac{1}{2}$	10	$12\frac{1}{2}$	15
Disinfectant					
1 : 700	—	—	—	—	—
1 : 800	+	—	—	—	—
1 : 900	+	+	—	—	—
1 : 1000	+	+	+	+	+
1 : 1100	+	+	+	+	+
Phenol					
1 : 80	—	—	—	—	—
1 : 90	+	—	—	—	—
1 : 100	+	+	+	+	—
1 : 110	+	+	+	+	—
1 : 120	+	+	+	+	+
1 : 130	+	+	+	+	+

$$\text{Coefficient} = \frac{\frac{700}{80}+\frac{900}{90}+\frac{1000}{110}}{3} = \frac{8.7+10.0+9.0}{3} = 9.2$$

REDDISH METHOD

In this test *Staphylococcus aureus* is used as the test organism. It is claimed that this test is more efficient as a germicidal index than the phenol coefficient method because it employs a larger quantity of broth culture, thereby requiring the germicide to act in the presence of considerable organic matter.

Culture Medium.—The culture medium used throughout the preliminary testing and the actual test is as follows:

Beef extract (Liebig's)....	0.5 per cent
Peptone (Armour's).	1.0 per cent
Sodium chloride. ..	0 5 per cent
Water (distilled)	q.s.

The P^H is adjusted to 6.6 to 6 8. Sterilization is accomplished by either fractional sterilization in the Arnold sterilizer or by autoclaving.

Test Culture.—At least three daily subcultures should be made using the above nutrient broth, before any tests are made. It is also essential that the strain of staphylococcus possess a certain resistance to phenol. To determine this a standard 5 per cent solution of phenol is used. Five dilutions are made, namely, 1 : 50, 1 : 60, 1 : 70, 1 : 80 and 1 : 90 and 5 c.c. of each placed in sterile test tubes. At intervals of thirty seconds, 0.5 c.c. of a twenty-four-hour broth culture of the staphylococcus is added to each and the tubes placed in a water bath at 20° C. At intervals of five, ten and fifteen minutes a 4 millimeter loopful is transferred to a test tube containing 10 c c. of the nutrient broth. All cultures are incubated at 37° C. for forty-eight hours and the results noted.

A staphylococcus strain of proper resistance must not be killed by the 1 : 70 dilution in ten minutes nor by the 1 : 80 dilution in fifteen minutes. Such strains may or may not be killed by the 1 : 60 dilution in five minutes and may or may not be killed by the 1 : 70 dilution in fifteen minutes. It is highly desirable, of course, that strains used show the maximum resistance, namely, resistance to 1 : 60 dilution for five minutes and to the 1 : 70 dilution for fifteen minutes.

Method.—1. Having determined that the particular culture of *Staphylococcus aureus* has sufficient resistance to phenol as outlined above, three successive daily cultures are made. For convenience five arbitrary dilutions of the germicide to be tested are made, or the first tube may contain the pure germicide and only four dilutions made. Five c.c. of each dilution are placed in large test tubes and held in a water bath at 37° C. At intervals of thirty seconds, 0.5 c.c. of the nutrient broth culture of staphylococcus is added to each dilution.

2. Each tube is shaken immediately and care must be exercised that no culture remains on the inside of the test tube that has not come in contact with the germicide. This may occur through careless manipulation of the pipet when adding the culture to the diluted germicide.

3. Several times during the test the tubes are shaken.

4. At intervals of five, ten and fifteen minutes, one 4 millimeter loopful of the germicide-culture mixture is transferred to tubes containing 10 c.c. of the sterile nutrient broth. These are incubated at 37° C. for forty-eight hours and observed for evidence of growth.

5. Where a compound of high germicidal activity is being tested, it has been recommended that transfers be made to tubes or flasks containing 50 to 100 c.c. or more of nutrient broth in place of 10 c.c. amounts. This would dilute any germicide carried over in culturing to a point where no growth-restraining properties would be manifested.

6. The dilution of germicide capable of killing the test organism in five minutes is considered efficacious when used in practice.

KOLMER BACTERIOSTATIC METHODS

This test is of extreme simplicity and yields sharply defined results. It determines the highest dilution of a disinfectant capable of restraining the growth of the test organism for a stated period of time and is of particular value for comparing the antiseptic properties of various chemical agents.

Method Employing Nutrient Bouillon.—For tests with staphylococci, *B anthracis, B. typhosus, B. coli* and such hardy organisms, plain beef extract broth with a P^H of 7.1 may be employed; Kligler has used a medium prepared of 1 per cent Fairchild's peptone, 0.5 per cent dibasic potassium phosphate, 0.5 per cent sodium chloride and 0.1 per cent glucose with a constant P^H of 7.1. The reaction of any medium employed is particularly important as the results may be greatly modified by this factor. For streptococci and pneumococci, hormone broth (Huntoon) with 0.1 per cent dextrose and a P^H of 7.7 is to be preferred, and for tubercle bacilli, the ordinary 5 per cent glycerin-hormone broth employed in the manufacture of Koch's tuberculin should be used. *It is always advisable to determine beforehand that the organism will grow well in the medium employed before the tests are conducted.*

1. As a general rule, ten dilutions are advisable and for this purpose ten sterile test tubes are arranged for each compound, including a set for the bichloride of mercury or phenol controls.

2. In all tubes except No. 1 of each series, place 1 c.c. of sterile distilled water.

3. In tubes 1 and 2 place 1 c.c. of the stock solution of disinfectant which is ten times higher than the final dilutions desired. Mix No. 2 and transfer 1 c.c. to No. 3 and so on to No. 10 from which discard 1 c.c.

4. In a flask of 99 c.c. of the culture medium, place 1 c.c. of a twenty-four to forty-eight hour broth culture of the test organism; with such organisms as streptococci and pneumococci it is well, however, to seed by mixing 5 c.c. of a broth culture with 95 c.c. of the culture medium. Mix well and add 9 c.c. to each tube of the set; the remaining 10 c.c. are placed in a sterile tube as a control on the culture.

5. In tests employing tubercle bacilli, it is better to add 9 c.c. of sterile medium to each tube and then to seed by floating a loopful of bacilli on the surface of each.

6. The final dilutions are now ten times higher in each tube; for example, 1 c.c. of 1 : 1000 stock solution in No. 1 becomes 1 : 10,000 and the final dilution in No. 10 is 1 : 5,120,000.

7. The tubes are incubated and the results recorded daily for five days. When the medium remains clear the result is recorded as *minus*; when a visible growth appears, the result is recorded as *plus*. At the end of this period the tubes may be cultured by transferring several loopfuls to slants of a solid medium to determine whether the organisms have been killed or merely restrained. In this manner a bactericidal test is conducted at the same time in which a few organisms have been exposed to the disinfectant for five days. The control should be subcultured at the same time to make sure that the organisms are viable.

8. The results are expressed according to the highest bacteriostatic and bactericidal dilutions and also according to the bichloride or phenol coefficients previously described.

9. After obtaining in this manner an approximate idea of the activity of the compound under study, a second series of dilutions is prepared in which the variations from tube to tube are less marked.

Method Employing Serum, Blood, Ascites Fluid or Muscle Extract.— These tests are conducted in exactly the same manner as described above except that to 89 c.c. of a suitable broth medium are added 10 c.c. of sterile serum, blood or ascites fluid; the mixture is then seeded with 1 c.c. of a broth culture of the test organism; with such organisms as the pneumococcus and streptococcus, however, it is generally advisable to use 85 c c. of both, 10 c.c. of serum, blood or ascites fluid with 5 c.c. of broth culture of the test organism. This gives a 10 per cent solution of serum, blood, or ascites fluid, and while more or less may be employed as desired, yet experience has indicated that the above is satisfactory for eliciting the influence of these substances upon the degree of antibacterial activity of disinfectants.

Numerous experiments have shown that while the bacteriostatic and bactericidal activity of various disinfectants is reduced to a greater extent on a 50 per cent than on a 5 per cent dilution of serum, defibrinated blood, or ascites fluid, yet for all practical purposes a 10 per cent solution is satisfactory for this purpose and in view of the large amounts required, is to be preferred from the standpoint of economy. With muscle extracts, however, it may be advisable to use equal parts with broth (50 per cent) but muscle extract may be prepared so cheaply and quickly, as required, that the questions of economy and supply are not involved. These preparations are likewise slightly cloudy but not usually to a degree sufficient for interfering with the ease and accuracy of readings. I may add in this connection, however, that a menstruum containing 10 per cent of a muscle extract prepared of ordinary beef or veal requires a far higher concentration of disinfectants than a menstruum of 10 per cent blood or serum; this is doubtless due to the presence of large numbers of various bacteria in addition to the test organism.

Other special media like hormone-dextrose broth with brain tissue, ascites broth with sterile kidney, etc., may be employed in tests of this kind, and while the results vary according to the constitution of the medium, yet if mercuric chloride or phenol are included in each and every test, the results may be expressed in terms of the coefficients or indices. The coefficients, however, will vary according to the chemical nature of the compound as this is influenced by the constitution of the medium; for example, some compounds of mercury, like mercurophen and metaphen, maintain a higher degree of bacteriostatic activity in a serum, blood, or brain medium than mercuric chloride and thereby yield higher coefficients. In other words, while the bactericidal activity of almost all disinfectants is reduced in the presence of serum, blood, muscle extract, etc., the degree of reduction varies considerably among different compounds.

Method Employing Solid Media; Mycostatic Test.—It is very easy to employ solid culture media in this technic and for such organisms as grow better on solid than fluid medium, as the various yeasts and molds, the former are to be preferred.

In conducting this test, a series of dilutions of the disinfectant in amounts of 1 c.c. in sterile distilled water are prepared in sterile test tubes as previously described. To each tube and a control are now added 9 c c. of an appropriate agar medium cooled to 42° to 45° C.; the contents are well mixed and allowed to harden in slants. The dilution in each tube is now ten times higher; or 4 c.c. of medium may be added to each tube, which renders the final dilution in each five times higher.

For such organisms as staphylococci, *B. typhosus, B. coli,* etc., plain 2 per cent agar (P^H 7.1) may be employed; for streptococci and pneumococci a hormone-dextrose (0.1 per cent) agar with a P^H of 7.7 is to be preferred. For tubercle bacilli, a glycerin agar may be employed since an egg medium may be unsatisfactory because heating for inspissation and sterilization may break up some disinfectants. For parasitic molds like *Trichophyton rosaceum, Microsporon audouini* and *Achorion schoenleinii,* Sabouraud's maltose medium titrated to + 1.0 to phenolphthalein, may be employed. Whatever medium is chosen, it must be adopted for cultivating the test organism, should be sterile, liquefied by heating, and added to the tubes containing varying dilutions of the disinfectant while still fluid after being cooled to 40° to 45° C. After hardening has occurred, each tube is inoculated in as uniform manner as possible and the tubes incubated for a period of five to ten days (mycostatic tests for two weeks or longer at room temperature) as decided upon. The results are expressed according to the highest dilution of disinfectant capable of preventing the growth of the test organism. Mercuric chloride or phenol controls may be included and the results expressed in terms of coefficients.

SECTION IV

SEROLOGICAL METHODS

CHAPTER XXIV

METHODS FOR THE COLLECTION OF BLOOD AND SERUM

COLLECTION OF BLOOD

Precautions.—1. Collection from a vein is advisable and the left arm is preferred.

2. Blood should not be taken immediately after a meal but any time after one hour is satisfactory. *It is not necessary for the patient to be fasting.*

3. As far as possible avoid drawing blood for the Wassermann test during febrile periods and acute alcoholism.

4. Sterile containers are advisable, although if the test is to be done within three days the presence of a few organisms is without effect.

5. Five c.c. of blood are sufficient. *When mailed a small container should be used in order that the contents shall reach almost to the stopper to avoid undue agitation and hemolysis.*

6. *Specimens for mailing should be shipped at once* in order to avoid undue hemolysis and anticomplementary sera. Under these conditions specimens can be shipped long distances.

7. If cotton stoppers are used, due care must be taken to prevent blood from coming in contact with them.

8. *Each specimen should be labeled at once* to prevent the very regrettable and inexcusable mistakes sometimes made. Labels may come off and should be held on with rubber bands.

From the Veins of Adults.—1. Place a tourniquet about the arm above the elbow; request patient to open and close the hand vigorously.

2. Cleanse the skin over a prominent vein with tincture of iodine and alcohol.

3. Remove 5 to 10 c.c. of blood with a sterilized Luer or Record syringe fitted with a No. 20 to 22 needle (Fig. 248). Transfer the blood to a small sterile test tube, vial or other suitable container.

4. The Keidel tube is particularly convenient. A modified Keidel tube made by Harris Comer, Philadelphia, enables one to see a flow of blood and,

FIG. 248.—SYRINGE METHOD FOR OBTAINING BLOOD.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)
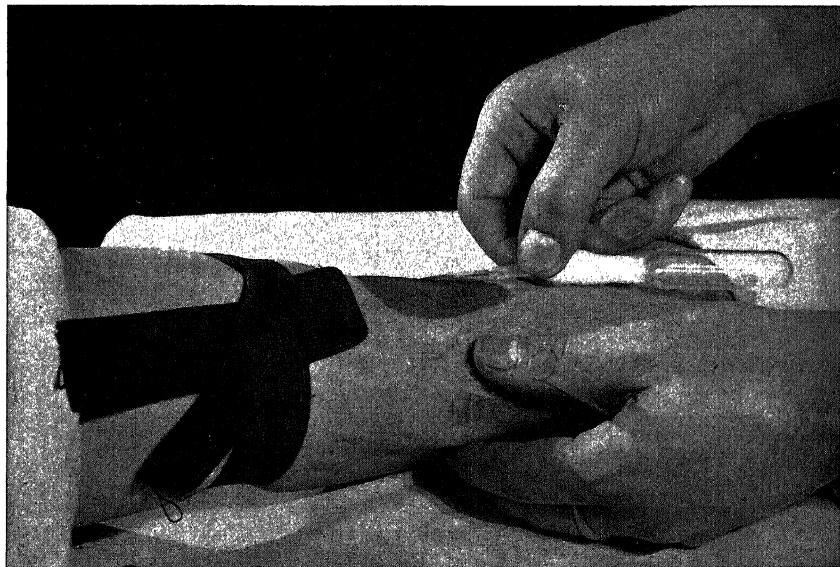
FIG. 249.—SECURING BLOOD WITH THE KEIDEL TUBE; METHOD OF PASSING THE NEEDLE.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

therefore, that the vein has been entered, before the neck of the vial is broken (Figs. 249 and 250). This tube is particularly convenient. After filling with blood, the ampule should be sealed by bending over the rubber stem with a rubber band to prevent loss if specimen is mailed.

5 Release the tourniquet and then withdraw the needle.

6. As a general rule, the wound requires no further attention unless several punctures have been made, in which case it should be cleansed with alcohol and covered with flexible collodion.

From a Finger.—1. Small amounts of blood for agglutination, Wassermann, transfusion, blood chemistry (micromethods) and other tests are easily obtained by puncturing a finger.

2. The hand must be warm with good circulation. If cold and clammy,

FIG 250—SECOND STEP IN SECURING BLOOD WITH THE KEIDEL TUBE
(From Kolmer, *Chemotherapy,* W B. Saunders Co)

have patient immerse the hand for a few minutes in hot water and rub briskly with a towel.

3. Cleanse tip of finger and Daland lancet (broad blade) with alcohol; dry.

4. Puncture across the lines at tip of finger.

5. Massage blood into a small test tube; with a large tube too much is lost on the sides (Fig. 251).

From Infants.—Blood may be obtained at birth by allowing the umbilical cord to bleed for about 5 c.c. into a container.

A few c.c. of blood may be readily obtained from infants by puncturing one of the large toes with a Daland lancet and massaging the blood into a small test tube, as described above for securing blood by puncture of a finger.

In the case of children from one to six years, sufficient blood may be obtained by puncture of one or several fingers In children over four years of age, a vein may be entered and blood drawn with a Keidel tube or syringe as above described.

Blood may be obtained with a syringe from one of the external jugular veins or from a temporal vein.
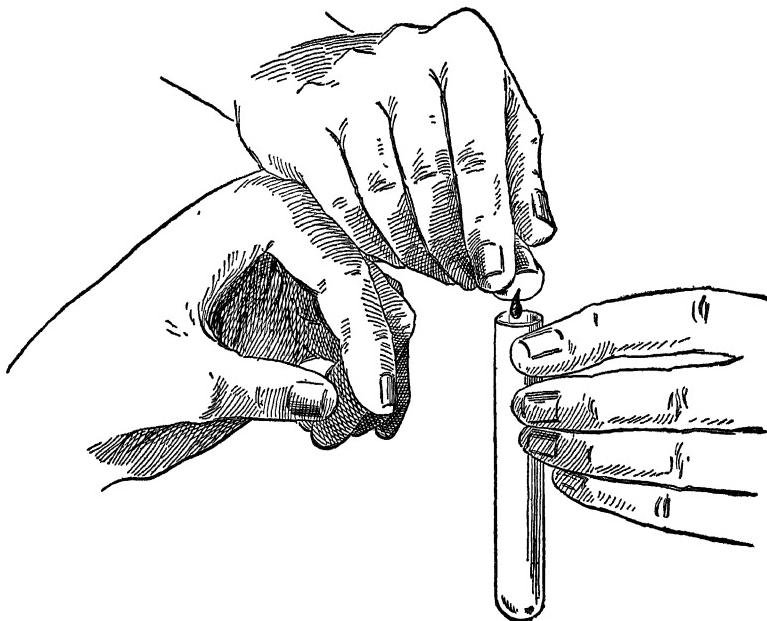
Fig 251 —Securing Blood from a Finger
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B Saunders Co)

Blood may be secured from infants under one year of age by puncture of the superior longitudinal sinus as follows:

1. The infant is wrapped in a blanket and the head is steadied by an assistant.

2. The puncture is made on the median line of the posterior angle of the anterior fontanel.

3. The skin is carefully cleansed The needle, gauge No. 18, with a short bevel, sterilized and attached to a sterile 5 c.c. Record or Luer syringe, is passed inward at a right angle for a distance of about 4 millimeters and suction made; if blood does not flow the needle should be passed about 2 millimeters farther, which suffices for the majority of children up to fifteen months of age

4. At least 3 to 5 c.c. of blood may be safely withdrawn and discharged into a vial. The puncture site is then cleansed and may be sealed with a touch of collodion.

SEPARATION OF THE SERUM

1. If serum is desired at once, allow the blood to coagulate for a few minutes, break up thoroughly with a rod and centrifuge.

2. If serum is not needed at once, place specimen in a refrigerator for spontaneous contraction of the clot and separation of serum. If this is unsatisfactory, break up the clot with a rod and centrifuge.

PRESERVATION OF SERUM

1. Sterile serum may be kept in sterile vials or other containers in a refrigerator without a preservative.

2 Otherwise it is well to add a preservative, as 0.5 per cent phenol or o 3 per cent tricresol (preferred). Keep a 5 per cent stock solution and add 0.05 c.c. to each c.c. of serum.

3. Rabbit serum hemolysins may be preserved by adding to the serum an equal part of chemically pure glycerin; mix well and keep in refrigerator. Hemolytic sera may be also dried on filter paper (Noguchi) and kept in refrigerator.

4. Immune sera (antitoxins, agglutinins) may be preserved in dried powder form.

5. Guinea-pig complement serum may be preserved for at least three weeks (sometimes longer) by adding 0.1 gram of pure sodium chloride to each c.c. and keeping on ice. For use, dilute 1 c c. with 9 c c. of water to give a 1 : 10 isotonic solution. For higher dilutions, dilute with saline (for 1 : 30 dilute 1 part of 1 : 10 with 2 parts of saline) Preserved complement loses first in fixability by antigen and antibody in syphilis before loss in hemolytic activity.

CHAPTER XXV

METHODS FOR CONDUCTING AGGLUTINATION TESTS

MICROSCOPIC AGGLUTINATION METHODS FOR TYPHOID AND PARATYPHOID FEVERS

1. Living cultures of typhoid and paratyphoid bacilli (A and B) are preferred and they should be of proper density and free of clumps (Figs. 252 and 253).

2. The cultures should be cultivated in tubes of broth at about 25° C. (on top of the incubator) for eighteen to twenty-four hours and give a good growth
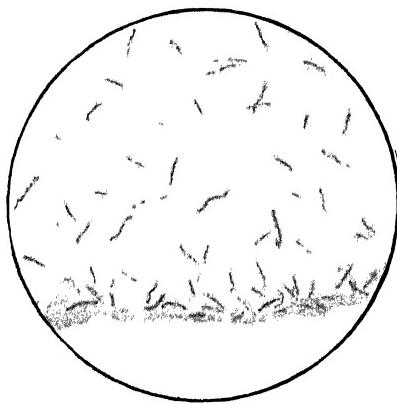
FIG 252 —A SATISFACTORY CULTURE FOR MICROSCOPE WIDAL TEST.

Note proper density and freedom from clumping. (From Kolmer, *Infection, Immunity and Biologic Therapy,* W B. Saunders Co)
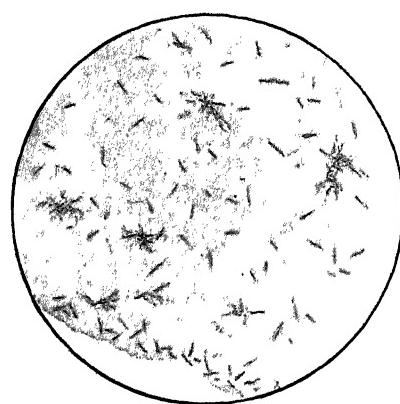
FIG. 253 —AN UNSATISFACTORY CULTURE FOR MICROSCOPE WIDAL TEST.

Note that it is too heavy and shows false clumping (From Kolmer, *Infection, Immunity and Biologic Therapy,* W. B Saunders Co)

of motile bacilli. Stock cultures on agar slants may be kept in a refrigerator and transplanted at intervals. When tests are frequently conducted the broth cultures should be subcultured daily.

3. When living cultures are employed, due care must be exercised and the slides, cover glasses, etc., placed in 5 per cent cresol or boiled for five minutes before handling and cleaning. The working table should be wiped with 5 per cent cresol.

Widal Test.—WITH DRIED BLOOD —1. Puncture finger or lobe of ear and collect a few drops of blood on a clean glass slide or paper and allow it to dry (do not heat to hasten drying).

2 Place a loopful of a twenty-four-hour culture of typhoid bacilli in the center of a clean cover glass.

3 Moisten the dried blood with a loopful of normal salt solution. Rub up the dried blood and transfer sufficient to the cover glass and mix thoroughly with the drop of culture. The mixture should have a delicate orange-pink color.

4. Mount the cover glass with vaselin on a concave slide, and allow it to stand one hour.

FIG 254—A POSITIVE WIDAL REACTION (Wood)

5. Prepare another slide in the same manner, using the culture only (control),

6. Make similar preparations with the cultures of paratyphoid bacilli.

7. Examine the slides with the 1/6 objective.

A positive reaction is indicated by clumping of the bacilli and more or less loss of motility as compared with the controls (Fig. 254). While this method is convenient for municipal laboratories it is not as accurate as methods employing known dilutions of serum and it is recommended, therefore, that the test be conducted with serum whenever possible.

WITH SERUM.—1. Prick finger and obtain 0 5 to 1.0 c.c of blood in a small test tube or fill a Wright's capsule (Fig. 255).

2. Draw off serum with capillary pipet (Fig. 256) after standing or centrifuging (serum should be clear and free of corpuscles).

3. Take two small watch crystals, hollow slides, or small test tubes and place one drop of serum in each.

FIG 255 —COLLECTING BLOOD IN A WRIGHT CAPSULE.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B Saunders Co.)

4. Add to one 24 drops of normal salt solution and to the other 49 drops, making dilutions of 1 : 25 and 1 : 50, respectively.

5 Place one loopful of an eighteen to twenty-four hour bouillon culture of typhoid bacilli in the middle of each of three cover glasses.

6. To the first, mix one loopful of serum diluted 1 : 25. To the second, mix one loopful of serum diluted 1·50. To the third, mix one loopful of normal salt solution.

7. Mount each in vaselin on hanging drop slide. The final dilutions obtained are 1 : 50 and 1 : 100; therefore mark the slides as follows: No. 1, 1 : 50; No. 2, 1 : 100; and No 3, control.

8. Make similar preparations with the paratyphoid cultures.
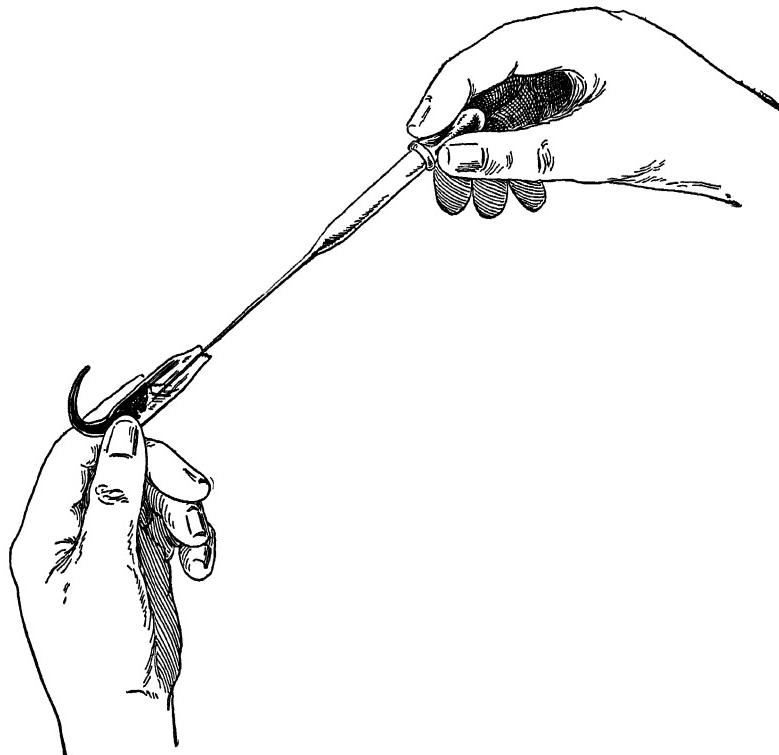
FIG 256—REMOVING SERUM FROM A WRIGHT CAPSULE
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W B. Saunders Co.)

9. Allow to stand at room temperature for one hour. More definite reactions may be obtained by placing the slides in an incubator at 37° C. for an hour.

10. Examine with 1/6 objective. Controls should be inspected first and should not show any clumping or loss of motility.

11. Examine the 1 : 50 and 1 : 100 dilutions for clumping and loss of motility.

12. Higher dilutions may be employed but the above are ordinarily sufficient.

MACROSCOPIC AGGLUTINATION METHODS FOR TYPHOID AND PARATYPHOID FEVERS

1. Prepare suitable suspension of the organism to be used in the test. The method of Dreyer is recommended, as follows:

(*a*) Inoculate a flask of bouillon with a known agglutinable strain of the organism.

(*b*) At the end of twenty-four to forty-eight hours, if a good growth is obtained, shake well and add sufficient formalin to make 0.1 per cent (1 c.c. commercial formalin to 1000 c.c. of culture). The suspension should contain about 2,000,000,000 bacilli per c c.

(*c*) Shake well and place in refrigerator at 6° to 8° C.

(*d*) Shake daily and make subcultures for sterility.

(*e*) When sterile (usually after three or four days) transfer to sterile bottles with rubber stoppers. Keep in a cold dark place.

2. If this antigen is not available, a good suspension may be prepared by washing off agar slant cultures with formalized saline solution (0 4 per cent in saline); shake with glass beads and filter through sterile paper. The suspension should be heavy enough for the tests; each c.c. should contain at least 2,000,000,000 bacilli. Or a good forty-eight to seventy-two hour broth culture may be employed after heating for an hour in a water bath at 60° C.

3. Secure at least 1 c.c. of blood by finger puncture or larger amounts from a vein; separate the serum.

4. Place seven small test tubes in a rack and mark 1 to 7. To tube 1 add 1.8 c c. physiological salt solution; to tubes 2 to 7 (inclusive) add 1 c.c. salt solution.

5. To tube 1 add 0 2 c c. of the patient's serum. Mix and transfer 1 c.c. to tube 2. Mix tube 2 and transfer 1 c.c. to tube 3. Repeat until tube 6 is reached. From tube 6 discard 1 c.c. Tube 7 is the culture control tube.

6. Add 1 c c. of the antigen to each of the seven tubes. This doubles the serum dilutions. The tubes now contain the following dilutions of serum: 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320, 1 : 640.

7. Shake the tubes and place in incubator at 37° C. for two hours. Remove from incubator and let stand at room temperature for several hours or in refrigerator overnight.

8. Make readings. Inspect control tube first; it should show uniform cloudiness. A positive reaction is shown by masses or clumps of bacteria which are broken up with difficulty; the supernatant fluid is clear (Fig. 257).

9. If the tests are conducted with the serum of an individual previously vaccinated with typhoid-paratyphoid vaccine and suspected of having typhoid fever, the test must be conducted every two or three days with the same antigen and with great technical care. A definite rise in agglutination titer indicates typhoid infection; if the titer remains unchanged, typhoid infection is probably not present.

Slide Test (Bass and Johns).—1. Prepare a suspension of typhoid bacilli containing 10,000,000 per c.c.; heat in a water bath at 60° C. for one hour
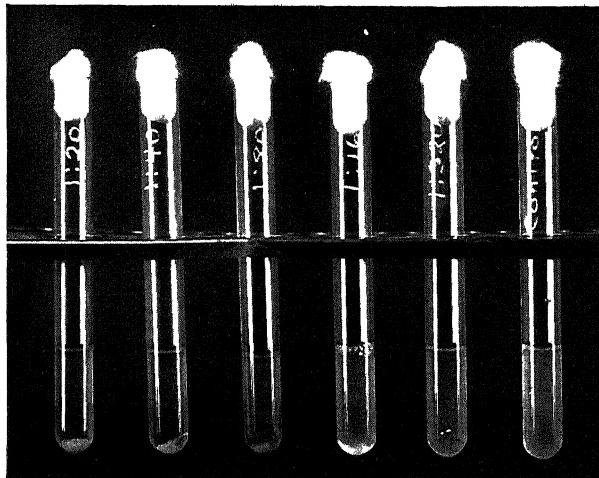
FIG 257.—POSITIVE MACROSCOPIC AGGLUTINATION REACTION
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B Saunders Co)

and preserve with 1 per cent formalin (to each 99 c c. add 1 c.c. of commercial formalin). Keeps several months if tightly corked. Shake before using.
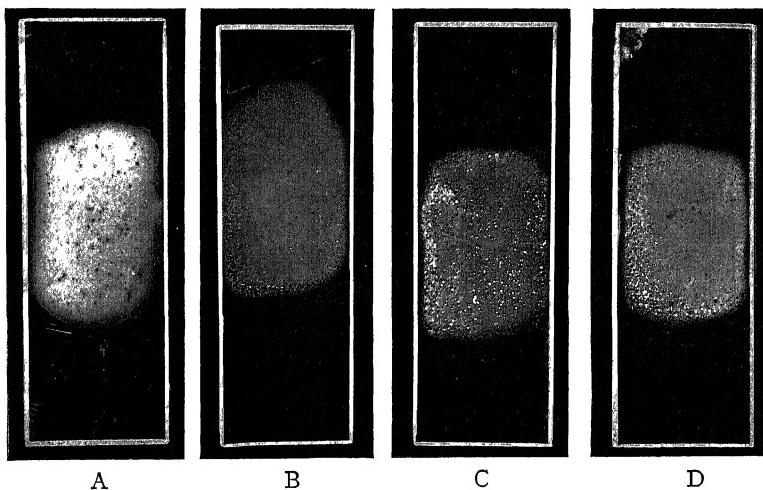
FIG 258—A SERIES OF AGGLUTINATION TESTS
A. Negative. B. Weakly positive C Moderately positive. D Strongly positive.
(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore)

2. Prepare a film of blood on a slide in the usual manner, using about one-quarter drop.

3. After drying, place one drop of water on the film and dissolve the blood with a toothpick or other suitable instrument.

4. Add one drop of the suspension of typhoid bacilli and mix with the blood by tilting from side to side and from end to end.

5. A positive reaction occurs in two minutes, with the formation of small grayish clumps and fine granular sediment (Fig. 258). Do not mistake dust particles for agglutination.

Absorption Test for Differentiating between Typhoid and Paratyphoid Fevers.—1. Arrange four rows of four small test tubes, each row to contain 1 c.c. of serum dilutions 1 : 20, 1 : 40, 1 : 80 and 1 : 160, respectively.

2. In each tube of the first and second rows emulsify five large loopfuls of typhoid bacilli. Add an additional control tube carrying 1 c c. of saline and culture.

3. In each tube of the third and fourth rows place paratyphoid bacilli (A or B as decided) ; add a control tube.

4. Mix gently and place in water bath at 37° C. for two hours. Record results.

5. Centrifuge all tubes except controls and transfer supernatant fluids to four more rows of tubes.

6. To each tube of the first and third rows add typhoid bacilli; to the second and fourth rows add paratyphoid bacilli. Mix well and place in water bath two hours at 38° C.

7. If typhoid fever is present, agglutination will be strong in the first and second rows of the first part of the test and practically unchanged in the third row of the second part.

8. If paratyphoid is present, agglutination will be strong in the third and fourth rows in first part of the test and practically unchanged in the fourth row of the second part. If paratyphoid B is employed with negative results, repeat the test with paratyphoid A.

HUDDLESON RAPID AGGLUTINATION TEST FOR UNDULANT FEVER OF MAN AND ABORTION DISEASE OF LOWER ANIMALS

According to prevailing opinion the cause of undulant fever of man and abortion disease of the lower animals is due to the same organism, known as *B. abortus* (*Brucella abortus*) of Bang and *M. melitensis* (*Brucella melitensis*).

The agglutination test by Huddleson and his coworkers described herewith affords a rapid and accurate method for diagnosis with human and bovine sera and is regarded as just as reliable and specific as slower methods; owing to the extremely small size of the organism, microscopic tests place quite a strain on the eyes. A second and slower method is described on page 443.

Apparatus.—1. Serological pipets of 0.2 c.c. capacity graduated to 0.01 c.c.

2. Two or three plates of double thickness window glass ruled with diamond-point into inch squares. A convenient size is 14 by 6 inches with 12

squares ruled horizontally by 5 squares vertically, leaving a blank margin of one-half inch around the bottom and top of the plate and one inch on the ends.

3. Dark-field illumination box (optional). This apparatus, while of unquestionable value for making readings, may be dispensed with if the glass plate is placed upon an ordinary laboratory table with a black background.

A convenient size box, 14 inches long by 9 inches wide and 8 inches deep, can be constructed from one-half inch material. One side of the top is covered to a width of 3 inches to protect the eyes of the operator from the lights which are placed just under the top extension piece near the ends. The inside
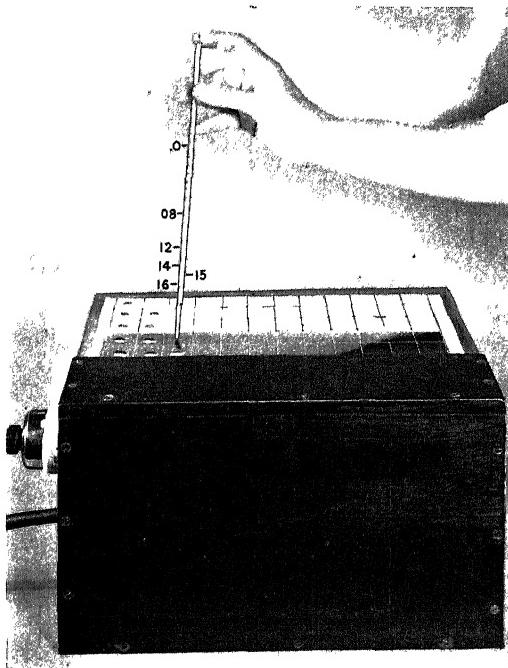
FIG 259.—AGGLUTINATION TEST FOR UNDULANT FEVER AND ABORTION DISEASE.
Placing serum (Huddleson.)

of the box behind the lights is painted white and the remainder black. This serves to produce a black background for objects placed on the plate which receive their illumination indirectly. A snap switch on the cord controls the lights (see Fig. 259).

4. Clean toothpicks for mixing serum and antigen on the glass plate.

Preparation of Antigen.—1. Inoculate Blake bottles containing liver infusion agar with a suspension of *Brucella abortus*. A single strain, the agglutinability of which has been previously determined by other tests, should be used.

2. Incubate the cultures for seventy-two hours at 37° C.

3 Wash off the growth with distilled water containing 12 per cent sodium chloride (C.P.) and 0.5 per cent phenol. Use as little solution as possible or otherwise the suspension will be too dilute and it will be necessary to centrifuge and remove some of the supernatant fluid. It takes much longer to remove the growth with this solution than with ordinary physiological saline (often as long as two hours).

4 Filter the suspension through a thin layer of absorbent cotton to remove pieces of the medium or other foreign material.

5. Add 0.01 c.c. of a saturated aqueous solution of gentian violet to each 100 c.c. of suspension. This is added to prevent the growth of organisms not inhibited by the phenol.

6. Place the suspension in a beaker and slowly boil for ten minutes.

7. Filter through a thin layer of absorbent cotton.

8. Cool rapidly and adjust the reaction to a P^H 6.8. The antigen is now ready to be standardized.

Standardization of Antigen.—1. Place 0.5 c.c. of antigen in each of five small test tubes and mark the tubes from 1 to 5.

2. Add 12 per cent saline solution to each tube as follows·

No 1 0 1 c c.
No. 2 . 0 2 cc.
No. 3: 0 3 c c
No. 4: 0 4 c c.
No. 5· 0 5 c c.

3. Thoroughly mix the contents of each tube.

4. Test each of the five dilutions against three types of sera, namely, one containing agglutinins in a titer of 1 : 500 or 1 : 1000, one with a titer of 1 : 25, and a negative serum.

5. The dilution which shows an absence of clumping with the negative serum, complete clumping in 0.08 c.c. of the serum with the titer of 1 : 25, and complete up to and including 0.004 c.c. of the serum with 1 : 500 titer, is the one properly concentrated for use. The antigen can now be diluted in the same proportion as that in the tube giving the proper reactions in this test.

Procedure.—1. Separate the sera from the clots and centrifuge if necessary to remove corpuscles. Use unheated.

2. *The dark-field box should be placed where it is not too intensely lighted, as too much light from above interferes with the indirect lighting of the plate from inside the box.* A desk lamp is of great advantage while the serum and antigen are being placed on the plate but it should be turned off when the tests are ready to be read so as not to interfere with the indirect lighting from the box. *Do not turn on the light in the box until ready to read the test as the plate will otherwise become too warm.*

3. Arrange the serum samples in a row parallel with the box. The glass

plate, with the etched squares upward, is placed over the opening of the box and the identification number of the serum sample marked with a wax pencil on either the top or bottom of the row of squares used.

4. With a clean 0.2 c.c. pipet, draw up serum from the first blood sample to the zero mark on the pipet. Beginning in the bottom left-hand square of the plate, place the following amounts of serum in the succeeding squares towards the top (see Fig. 259):

		Reading on Pipet
1st square (0.08 c c.)	..	0.08
2nd square (0 04 c.c.)		0.12
3rd square (0 02 c.c)		0.14
4th square (0 01 c.c)		0.15
5th square (0.004 c.c.)		about midway, 0.15 and 0.16

5. This manner of placing the serum brings the smallest amount farthest from the heat of the electric bulb, reducing the rapidity of drying of the smallest amounts of serum. The procedure is continued, using the next set of vertical squares and a separate pipet for each sample. The best results are obtained by testing only four or five samples at a time, as otherwise the small amounts of serum dry out too much before the test is completed.

6. If the pipet has been placed deep in the serum, there will be some serum which will collect on the outside at the tip. For accuracy, this should be removed by touching the tip of the pipet against the lip of the vial.

FIG 260—AGGLUTINATION TEST FOR UNDULANT
FEVER AND ABORTION DISEASE.
Adding antigen   (Huddleson)

7. After thoroughly shaking the vial, remove a dropper full of the antigen. Holding the dropper in a vertical position, add one drop to each amount of serum on the plate. *Care should be taken to hold the dropper in a vertical position since holding it at another angle will make a considerable difference in the amount of antigen delivered. Always replace the dropper directly in the vial of antigen* (Fig. 260).

8. The final dilutions are now 1:25, 1:50, 1:100, 1:200 and 1:500.

9. With a clean toothpick mix the serum and antigen, using a new toothpick for each sample. Always start at the top of the plate in the square containing

the smallest amount (0.004 c.c.) of serum and continue downward to the largest amount. Spread the mixture over about three-fourths the area of the square without coming in contact with the etched dividing lines.

10. Immediately after the samples have been mixed, remove the plate from the box and tilt slightly backward and forward slowly for about two minutes. Place the plate on the box, turn on the light and record the results. When working without the box, make the readings against a light so that the plate is illuminated from beneath.

11. The reactions stand out very clearly as shown in Figure 261. It is not difficult to distinguish between complete clumping of the antigen and different degrees of incomplete clumping, e g., in sample 6 there is complete agglutination in all amounts of serum, while in sample 4 the clumping is complete in only the last three amounts. A negative serum (sample 1) causes no flocculation of the antigen. There are often encountered, however, sera which produce a trace of flocculation in the 0.08 c.c. amount. In the "slow" or test tube method, this occurrence will often pass unobserved unless viewed with a hand lens. This type of clumping appears to be due to the presence of native agglutinins in the serum (Fig. 261).

Fig. 261 —Different Degrees of Agglutination Reactions for Undulant Fever and Abortion Disease. (Huddleson.)

12. Immediately after using, the pipets should be rinsed several times with fresh water until thoroughly clean. Then boil in distilled water and drain all the water out before using again. The glass plate may be cleansed with cleaning powder and brush, after which it is rinsed with distilled water and dried. By having several clean plates available, one can proceed with the testing of additional samples without delay. *Absolute cleanliness of glassware is essential.*

Interpretation.—1. With human sera a positive reaction in a titer of 1 : 100 is considered diagnostic of undulant fever.

2. With cattle sera a positive reaction in a dilution of 1 : 50 is suspicious of present or past infection, while positive reactions in 1 : 100 or higher are diagnostic.

3. Agglutinins generally appear in the blood after the infection has been present for a week or longer.

Method for Testing Milk Serum.—This test can be conducted with milk from cows for the detection of those carrying *Brucella abortus* in the udders.

1. Collect separate samples of the fore milk from each quarter, using clean, sterilized vials of about one-half ounce capacity. Before collecting the samples, place in each vial a small amount of rennin powder (about what can be picked up between the prongs of a small pair of forceps or on the tip of a small knife blade). Then draw the milk directly into the vial and place in a slanting position. The serum will separate out in about an hour at room temperature.

2. The test is conducted with the milk sera in exactly the same manner as previously described for blood sera.

SECOND METHOD OF AGGLUTINATION TEST FOR UNDULANT FEVER OF MAN AND BOVINE INFECTIOUS ABORTION

1. Prepare antigen from four- to seven-day, glycerin-agar or liver infusion agar (preferred) cultures of several strains of *Brucella abortus* (Bang), washed off with and suspended in carbolized saline solution (1 part carbolic acid to 200 parts normal saline solution). Shake well to secure an even suspension and dilute as necessary to give a suspension of such density that ordinary book print is clearly read through 2 c.c. amounts in test tubes of five-eighths by five inches or the antigen may be prepared by cultivating a suitable strain on broad slants of liver infusion agar for forty-eight hours and removing the growths with 0.4 per cent formalin in saline solution. The suspension is filtered through sterile paper and tested for sterility after four days, the cultures on liver infusion agar being incubated for seven days. The sterile suspension is now centrifuged and the clear supernatant fluid discarded. The sediment is taken up in a small amount of 0.4 per cent formalin and the packed organisms kept in a refrigerator, dilutions of the desired density being prepared at the time the agglutination tests are conducted.

2. Use sera unheated.

3. For each serum arrange four test tubes (five-eighths by five inches) and place following amounts: 0.1, 0.04, 0.02 and 0.01 c c.

4. To each tube add 2 c.c. of antigen. The final dilutions are now 1:20, 1:50, 1:100 and 1:200.

5. Prepare a control of 0.1 c.c. of saline and 2 c.c. of antigen.

6. Mix well and incubate at 37.5° C. for twenty-four hours.

7. The readings are made as follows:

> Positive with cattle sera: complete agglutination 1:50 or over; with human sera a positive reaction requires agglutination at 1:100 or over
>
> Weakly positive with cattle sera: partial agglutination of 1:50
>
> Suspicious with cattle sera: complete or partial agglutination in 1:20 only
>
> Negative with cattle sera: no agglutination in 1:20 or over

AGGLUTINATION TEST FOR TULAREMIA

1. Prepare antigen as follows:

(a) Use Blake bottles containing blood glucose cystine agar. Before inoculation, the bottles should dry several days in an inverted position in the incubator and any water of condensation present should be pipetted off. Inoculate each bottle with the entire growth from a blood glucose cystine agar slant of *B. tularense* No. 38, suspended in 1 c c. of physiological salt solution. Spread the suspension quickly over the surface of the medium in the Blake bottle by rocking the bottle in the hands.

(b) Incubate the bottles in their normal noninverted position at 37° C. for three days.

(c) Take off the growth of each bottle in 15 c.c. of physiological salt solution containing 0.2 or 0.3 per cent of formalin (U. S. P., strength 37 per cent). Throw down the bacterial mass by centrifugation, thereby washing the organisms. Pour off the supernatant fluid. Take up the bacterial mass in a small amount of formalized salt solution. This concentrated stock suspension has been found to be entirely reliable for agglutination tests even after storage for two years in the cold room.

(d) At the time of use dilute a portion of the concentrated suspension to the desired turbidity corresponding to No. 4 of the nephelometer (see Chapter XX).

2. Separate serum from blood specimen.

3. Prepare the following dilutions of serum in physiological salt solution: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 as described above for the undulant fever test.

4 Tube No. 7 is the culture control.

5. It is also recommended to set up at the same time and in the same manner a positive control set, using rabbit antitularense or positive human serum.

6. It is also recommended to set up tests with the patient's serum and antigens of *B. typhosus, B. paratyphosus* A and B and *B. melitensis* (*B. abortus*).

7. Incubate the tests for two hours at 35° to 37° C. Remove from incubator and let stand in refrigerator overnight.

8. Examine for agglutination. A definite agglutination in the 1 : 80 dilution is considered diagnostic.

AGGLUTINATION TEST FOR TYPHUS FEVER (WEIL-FELIX REACTION)

1. Prepare antigen by suspending twenty-four-hour agar cultures of *B. proteus x 19* in saline solution corresponding in density to No. 2 of the nephelometer (see Chapter XX).

2. Secure patient's blood and separate the serum.

3. Arrange seven small test tubes in a row and label.

4. Into No. 1 place 1.8 c.c. of saline solution and 1 c.c. into Nos. 2 to 7.

5. Into No. 1 place 0.2 c.c. of serum; mix well and transfer 1 c.c. to No. 2; mix well and transfer 1 c.c. to No. 3 and so on to No. 6, from which discard 1 c.c.

6. Add 1 c.c. of antigen to all tubes. The final dilutions are now 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640.

7. No. 7 is a control and receives no serum.

8. At the same time set up duplicate tests, employing a known positive human or rabbit immune serum; also tests employing a known normal human serum.

9. Incubate in a water bath at 45° C. for two hours.

10. Examine for agglutination. The reaction is usually apparent at the end of two hours.

11. Make final reading after tests have stood in the refrigerator overnight

12. Definite agglutination in 1:100 or higher is considered diagnostic.

AGGLUTINATION TEST FOR GLANDERS OF HORSES

1. Prepare antigen of an agglutinable strain of *Pfeifferella mallei* (*B mallei*). Only a small percentage of cultures are suitable.

2. Cultivate on glycerin agar with P^H 6.8 for twenty-four to forty-eight hours.

3. Wash off with sterile saline solution. Shake well to break up clumps. Dilute to proper density with saline. Heat at 65° C. for one hour. Add formalin to give a final 0.25 per cent. Keep tightly stoppered in a refrigerator.

4. Use sera unheated.

5. For each serum arrange six small test tubes and place 1 c.c. of saline in each.

6. Prepare a 1:50 dilution of serum (0.1 c.c. + 4.9 c.c. saline) and place 1 c.c. in No. 1. Mix well and transfer 1 c.c. to No. 2; mix and transfer 1 c.c to No. 3; mix and discard 1 c.c. Prepare a 1:250 dilution (1 c.c. of 1:50 + 4 c.c. saline) and place 1 c.c. in No. 4; mix and transfer 1 c.c. to No. 5; mix and discard 1 c.c. The dilutions are now 1:100, 1:200, 1:400, 1:500 and 1:1000. The sixth tube is the control.

7. Add 1 c.c. of antigen to all tubes, which doubles the dilutions.

8. Mix and incubate at 37.5° C. for twenty-four to forty-eight hours.

9. The reactions are interpreted as follows:

> Positive: complete agglutination 1:1000 or higher
> Suspicious: agglutination in 1:800
> Negative: no agglutination at all or partial agglutination 1:200 and
> 1:400

AGGLUTINATION TEST FOR BACILLARY WHITE DIARRHEA OF CHICKENS

1. Cultivate *Salmonella pullorum* on agar for forty-eight hours. Wash off with phenolized saline solution. Shake well and filter through cotton. Dilute to proper density. To each 100 c c. add 2 c.c. of 2 per cent solution of sodium hydroxide as recommended by Mathews for the prevention of precipitation, giving the cloudy reactions which may occur with as high as 75 per cent of sera.

2. The sera should be clear and as free as possible of hemoglobin.

3. For the test arrange four small test tubes.

4. Place 1.8 c.c. of saline in No. 1 and 1 c.c. in Nos. 2, 3 and 4.

5. To No. 1 add 0 2 c.c. of serum; mix and transfer 1 c.c. to No. 2; mix and transfer 1 c.c. to No. 3; mix and discard 1 c.c. No. 4 receives no serum and is the antigen control.

6. Add 1 c c. of antigen to all tubes to give final dilutions of 1 : 20, 1 · 40 and 1 : 80.

7. Mix well   Incubate at 37.5° C. for twenty-four hours

8. The results are interpreted as follows:

   Positive: agglutination in 1 · 40 or higher
   Suspicious: partial agglutination in 1 : 20
   Negative: no agglutination in 1 : 20

AGGLUTINATION TEST FOR FOWL TYPHOID

1. Cultivate *Salmonella gallinarum* (*B sanguinarium*) on agar for two or three days. Wash off the growths with phenolized saline solution and shake well. Filter through cotton and dilute to proper density. To each 100 c.c. may be added 2 c.c. of 2 per cent solution of sodium hydroxide for aid in preventing the precipitation sometimes yielding the cloudy reactions observed with fowl serum.

2. The sera should be fresh and as free as possible of hemoglobin.

3. For each serum arrange six small test tubes. Into No. 1 place 2 4 c.c. saline and 1 c.c. in the remaining tubes.

4. To No. 1 add 0 1 c.c. of serum. Mix, transfer 1 c.c. to No. 2 and discard 0.5 c.c. Mix No. 2 and transfer 1 c.c. to No. 3; mix and transfer 1 c.c. to No 4; mix and transfer 1 c.c. to No. 5; mix and discard 1 c.c. No. 6 is the antigen control and receives no serum.

5 To each tube add 1 c c. of antigen. The final dilutions are now 1 : 50, 1 : 100, 1 : 200, 1 : 400 and 1 : 800.

6. Mix well and incubate at 37.5° C. for twenty-four hours.

7. The results are interpreted as follows:

   Positive: agglutination 1 : 200 or higher
   Suspicious: partial agglutination in 1 : 50 to 1 : 100 but not higher
   Negative: no agglutination 1 : 50 or higher

METHODS FOR CONDUCTING BLOOD TRANSFUSION TESTS

Principles.—1. The serum of a patient (recipient) may agglutinate or hemolyze the corpuscles of a donor or the corpuscles of the patient may be agglutinated or hemolyzed by the serum of a donor. Blood transfusion requires therefore the use of compatible blood, when the latter is to be administered by intravenous injection.

2. This phenomenon is due to the fact that there are two main agglutinins (*a* and *b*) occurring in the serum and two main agglutinogens (*A* and *B*) occurring in the red blood corpuscles of human beings which permit placing the blood of an individual in any one of four main groups. Considerable confusion, however, and even accidents, have been caused by different arbitrary numberings or classifications, the three best known being as follows:

Moss	Jansky	Landsteiner	Per Cent of Approximate Occurrence in Adults
I	IV	*AB*	10
II	II	*A*	40
III	III	*B*	7
IV	I	*O*	43

3. If both agglutinogens are present in the corpuscles the serum is free of both agglutinins and the individual belongs to group *AB* or Moss I or Jansky IV. If the corpuscles contain neither agglutinogen the serum contains both agglutinins and the blood belongs to *O* or Moss IV or Jansky I. If the corpuscles contain agglutinogen *A*, the serum contains agglutinin *b* and the blood belongs to group *A* or Moss II or Jansky II. If the corpuscles contain agglutinogen *B,* the serum contains agglutinin *a* and the blood belongs to group *B* or Moss III or Jansky III. In other words, the serum regularly contains the agglutinins active for the absent agglutinogens; that is, corresponding agglutinins and agglutinogens do not coexist in one blood.

Precautions and Sources of Error.—1. *It is to be especially noted that group I of Moss corresponds to IV of Jansky and that IV of Moss corresponds to I of Jansky If blood grouping is reported by numbers, it is essential therefore always to state whether the Moss or Jansky classification is being used, in*

order to avoid serious accidents, or both of these may be dropped and the Landsteiner classification employed

2. For blood transfusion the patient's blood may be typed and a donor selected belonging to the same group But direct matching is recommended in addition, especially if the patient belongs to group *A,* because of the existence of subgroups and atypical agglutinins, as well as of donors with unusually high agglutinin titers. However, it is not quite safe to depend on direct matching alone because in cases of weak agglutinins incompatibility may escape attention. In other words, *of the two methods for selecting donors direct matching is preferred but it is better and therefore recommended that the patient be typed and direct matching tests conducted with donors belonging to the same group for the final selection of a donor.*

3. The tests should be done at 37° C. or room temperature to avoid errors due to "cold" or auto-agglutinins operative at low temperatures.

4. Pseudo-agglutination is a possible source of error readily avoided by dilution.

5. In microscopical tests, rouleaux formation of corpuscles is a possible source of error, especially for inexperienced workers.

6. A more important source of error lies in the fact that sera differ in their agglutinating titer from time to time, especially in the case of group *B* serum Patients receiving repeated transfusions may undergo obscure changes and especially require very careful direct matching tests for the selection of donors.

7 Diet may have an influence.

8 Especial care must be exercised in matching the blood of infants under two years because the group characteristics may not be fully developed

METHODS FOR DIRECT MATCHING

Microscopic Agglutination Tests.—1. Prepare finger of recipient and make deep puncture.

2 Collect 10 to 15 drops of blood in a small test tube containing 1 c c. of 2 per cent sodium citrate solution.

3 At the same time secure 1 to 2 c.c. of blood in a dry test tube.

4 Secure blood from the donor or donors in the same manner.

5 Break up the clots and centrifuge along with the tubes containing the citrated blood

6. Remove the supernatant fluids from the citrated tubes and add to the corpuscles in each tube 2 volumes of normal salt solution; suspend the corpuscles

7. Hanging drop preparations are prepared as follows:

(*a*) Two loopfuls of patient's serum plus one loopful of donor's corpuscles are mixed on cover glass and mounted with vaselin on a hanging drop slide.

(b) Two loopfuls of donor's serum plus one loopful of patient's corpuscles are mixed and mounted.

(c) Prepare similar preparations with the sera and corpuscles of additional donors

(d) Two loopfuls of saline and one loopful of patient's corpuscles are mixed and mounted (control)

(e) Two loopfuls of saline plus one loopful of donor's corpuscles are mixed and mounted (control)

(f) *Be sure to label the slides and do not trust to memory*

8. Allow to stand for fifteen minutes at room temperature.

9 Examine with 2/3 objective Controls should be examined first (step 7, d and e) and should not show any agglutination. False clumping and rouleaux formation at

FIG 262 —FALSE AGGLUTINATION
(From Kolmer, *Infection, Immunity and Biologic Therapy,* W B Saunders Co)

the margin of the mixture should not be mistaken for a positive reaction (Figs. 262, 263 and 264).

10. Agglutination in (a) of step 7 shows that the patient's serum is incom-
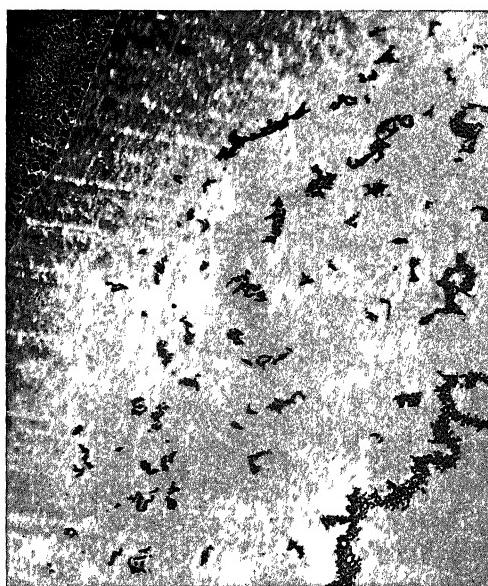
FIG 263 —TRUE AGGLUTINATION
(From Kolmer, *Infection, Immunity and Biologic Therapy,* W B Saunders Co.)

patible with the donor's corpuscles. Agglutination in (*b*) shows that the donor's serum is incompatible with the patient's corpuscles.

11. This direct test is always advisable before transfusion even if the patient is first grouped and a donor of the same group is selected in order to guard against the possibility of transfusing with an incompatible subgroup blood.

An alternative method for setting up the above tests is as follows:
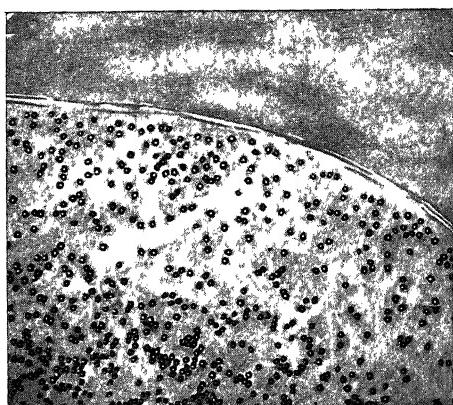
FIG 264—A NEGATIVE REACTION

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W B Saunders Co)

1. Prepare capillary pipets of approximately equal caliber for the corpuscles and serum of the recipient and each donor.

2. Arrange two clean slides for each test and proceed as follows:

(*a*) At the end of slide No. 1 mix a drop of the patient's corpuscles with a drop of donor's serum and a drop of saline solution. At the other end mix a drop of the patient's corpuscles with a drop of saline solution (this is a control on the corpuscles).

(*b*) At the end of slide No. 2 mix a drop of the donor's corpuscles with a drop of the patient's serum and a drop of saline. At the other end mix a drop of the donor's corpuscles with a drop of saline solution (control).

(*c*) Repeat the set-up in the same manner with the corpuscles and serum of each donor.

3. Rock the slides occasionally for about five minutes. If agglutination is not visible, place a cover glass over each mixture and let stand at room temperature for fifteen minutes. Examine each microscopically.

Macroscopic Agglutination Test (Brice).—1. This test may be used for more rapid testing of large numbers of donors and is conveniently conducted with a special plate (Fig. 265).

FIG. 265.—BRICE BLOOD TYPING PLATE.

2. Corpuscles and sera of the patient (recipient) and each donor are prepared as described above.

3. Mix 1 drop of the recipient's corpuscles with 4 drops of the serum of each of the donors, and a drop of each of the donors' corpuscles with 4 drops of the serum of the recipient, with a glass stirring rod, each in its proper place on the typing plate.

4. Lift the plate and oscillate with a rocking motion, which keeps all of the

corpuscles in movement, for seven minutes over a white background or transmitted light for evidences of agglutination.

5. The microscopical tests are more sensitive and to be preferred.

Macroscopic Agglutination and Hemolysis Tests (Kolmer).—1. Collect blood in citrate solution and in a small dry test tube from the patient and donor as just described.

2. Prepare the corpuscles and secure the sera as described.

3. Arrange four small test tubes and place 1 c.c of saline in each.

4. Into No. 1 place 5 drops of patient's serum and 1 drop of donor's corpuscles.

5. Into No. 2 place 5 drops of donor's serum and 1 drop of patient's corpuscles.

6. Into No. 3 place 1 drop of patient's corpuscles (control).

7. Into No. 4 place 1 drop of donor's corpuscles (control).

8. Mix each tube and place in a water bath at 37° C. for one hour.

9. Read the results. Tubes 3 and 4 should show no hemolysis or agglutination.

10. If hemolysis and agglutination do not occur in tubes 1 and 2 the bloods are compatible. If the patient's serum is incompatible with the donor's corpuscles, a positive reaction will occur in tube No. 1; if the donor's serum is incompatible with the patient's corpuscles, a positive reaction will occur in tube No. 2.

11. Repeat in same manner with the serum and corpuscles of each donor.

12. This test is recommended especially for hemolysis and not for routine work.

METHODS FOR BLOOD GROUPING

Method of Landsteiner.—1. Secure a few drops of blood in 1 or 2 c.c. of saline solution to give a 2 to 5 per cent blood suspension.

2. Into a small test tube (7-millimeters diameter) place a drop of type II (*A*) serum, a drop of saline solution, and a drop of the blood suspension.

3. Prepare a second tube in the same manner with a drop of type III (*B*) serum.

4. Shake the tubes several times and allow to stand at room temperature for fifteen minutes.

5. Remove a drop of each mixture with a thin glass rod to glass slides and examine microscopically with low magnification.

6. Positive reactions generally occur within a few minutes but in order to detect unusually feeble reactions, the negative reactions should be examined after one hour. Control tests with known cells *A* and *B* should be included. Special care must be taken to select test sera of known high agglutinating power. Sterile test sera can be kept preferably in the ice box or may be stored with a preservative (*e.g.*, chloroform) as described above

7. The readings and interpretations are made as described above.

Second Microscopic Method.—1. Prepare finger and make puncture of the individual to be grouped.

2. Collect 2 or 3 drops of blood in small test tube containing 1 c.c. of normal salt solution (approximately a 5 per cent suspension).

3. Prepare four capillary pipets of equal calibers, one each for group II (*A*) serum, group III (*B*) serum, corpuscle suspension and saline solution.

4. Arrange three clean slides.

5. On one slide, place a drop of II (*A*) serum and add a drop of corpuscles and saline solution; mix well.

6. On the second slide, place a drop of III (*B*) serum and add a drop of corpuscles and saline solution; mix well.

7. On the third slide mix a drop of corpuscles with a drop of saline solution (control).

8. Rock each slide occasionally for about five minutes to accelerate agglutination.

9. Place a cover glass on each and stand at room temperature for fifteen to twenty minutes.

10. Examine under microscope with low-power objective.

11. The control should show no agglutination.

12. The group is determined as follows:

(*a*) No agglutination with either groups II (*A*) or III (*B*) serum=group I of Jansky, group IV of Moss or group *O* of Landsteiner.

(*b*) Agglutination with both groups II (*A*) and III (*B*) serum=group IV of Jansky, group I of Moss or group *AB* of Landsteiner.

(*c*) Agglutination with group II (*A*) but no agglutination with group III (*B*) serum=group III of both Jansky and Moss or *B* of Landsteiner.

(*d*) Agglutination with group III (*B*) but not with group II (*A*) serum =group II of both Jansky and Moss or *A* of Landsteiner.

13. False clumping and rouleaux formation at the margin of the mixture should not be mistaken for a positive reaction.

14. Known group II (*A*) and group III (*B*) sera should be kept in a refrigerator preserved with chloroform or 0.5 per cent phenol. *The sera should be of such potency as to produce marked agglutination in one minute in final dilution of 1:8 or higher.* It is well to replenish every two or three months and frequent tests should be made to make sure that the sera are potent. It is easy to replenish the sera by typing the corpuscles of blood specimens submitted for the Wassermann tests. If corpuscles belonging to group II (*A*) are found, the excess serum may be preserved (group II or *A*); if corpuscles belonging to group III (*B*) are found, the excess serum should be preserved (group III or *B*). These two sera suffice for grouping by the Jansky, Moss or Landsteiner classifications. Group I (*AB*) and group IV (*O*) sera are unnecessary.

CHAPTER XXVII

METHODS FOR CONDUCTING COMPLEMENT-FIXATION TESTS FOR SYPHILIS AND BACTERIAL DISEASES

KOLMER COMPLEMENT-FIXATION TEST FOR SYPHILIS [1]

Glassware and Apparatus.—1. Pipets:

> 1 c.c. graduated in 0.01 c.c. to tip (Fig. 266)
> 5 c.c. graduated in 0.10 c.c.
> 10 c.c. graduated in 0.50 c.c.
> 14.4 c.c. graduated in 1.20 c.c.

An automatic pipet is highly recommended for rapid work and saving of time for pipeting saline solution, complement, antigen and hemolysin in the conduct of the tests.
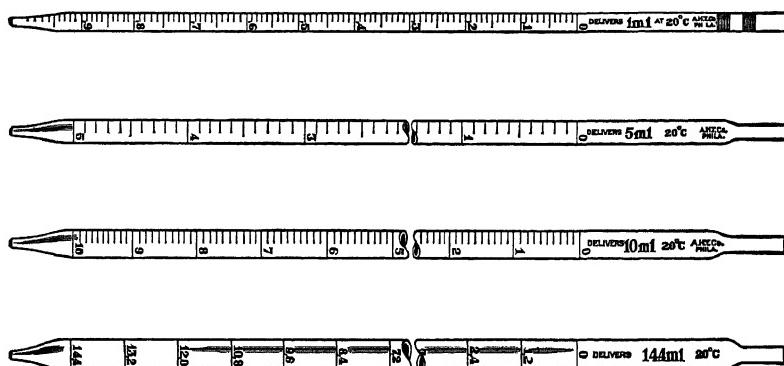
FIG. 266.—KOLMER SEROLOGICAL PIPETS

2. Test tubes: 85 by 13 millimeters (inside diameter) with rounded bottom and no lips.

3. Cylinders: glass-stoppered, graduated (50 or 100 c.c. capacity) to be used for measuring amounts over 50 c.c.

4. Centrifuge tubes: special graduated Kolmer centrifuge tubes of two sizes, 10 c.c. and 30 c.c. capacity (Figs. 267 and 268).

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[1] For a more detailed description of this test, including complement-fixation tests for the identification of sera, blood stains, seminal stains and for the detection of meat and milk adulteration, consult Kolmer's *Serum Diagnosis by Complement Fixation.* Lea and Febiger, Phila., 1928.

5. Test tube racks: galvanized wire racks carrying twelve rows of six tubes each (Fig. 269).

6. Water bath: any easily regulated water bath is suitable, or a simple galvanized pan carrying water to a depth of 8 centimeters can be used satisfactorily at 55° or 37° C. (Figs. 270 and 271).

7. Refrigerator: any refrigerator maintaining a temperature of 6° to 8° C. is satisfactory.

METHOD OF CLEANING GLASSWARE.—1. All glassware should be chemically clean and preferably sterile To clean tubes and flasks, empty and rinse in running tap water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in the hot air oven at about 160° C.

FIGS 267, 268 —KOLMER CENTRIFUGE TUBES

2. Pipets should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean pipets, rinse thoroughly in
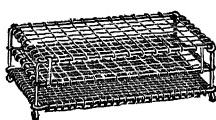
FIG. 269 —KOLMER RACK
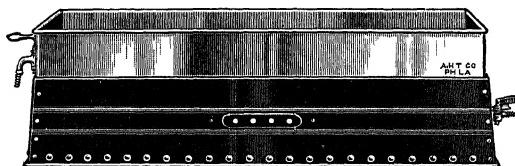
FIG 270.—KOLMER SEROLOGICAL BATH, GAS HEATED

running tap water, place in a metal box or wire basket and sterilize in the oven.

3. Flasks should be plugged with cotton and sterilized in the oven for thirty minutes at 160° C.

4. If glassware becomes cloudy, immerse in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid, and 25 parts water) for twenty-four hours. Rinse thoroughly in running tap water and proceed with the washing as before.

Preparation of Reagents. —SALINE SOLUTION.—Dissolve

FIG 271.—KOLMER SEROLOGICAL BATH, ELECTRICALLY HEATED.

8 5 grams of dry, chemically pure sodium chloride in 1000 c.c. of tap or distilled water. If the salt has absorbed moisture it should be dried in the hot

air oven for ten or fifteen minutes before weighing. Filter solution through paper into a flask fitted with a gauze-covered cotton stopper. Sterilize by heating in an Arnold sterilizer for one hour before use.

A satisfactory saline solution should not of itself be hemolytic when 1 or 2 drops of washed corpuscles are added to 5 c.c. of the solution in a test tube followed by water bath incubation for one hour. Neither should it be anti-hemolytic as determined by the hemolysin or complement titrations. The antisheep hemolysin should give a unit of at least 0 5 c c. of a 1 : 4000 dilution when titrated with 0.3 c c of 1 : 30 complement and 0.5 c.c. of a 2 per cent suspension of washed sheep corpuscles with a water bath incubation of one hour. It is possible that failure of hemolysis may be due to defective hemolysin or complement or to the use of corpuscles of increased resistance to serum hemolysis, but whenever these factors may be excluded it is likely that the saline solution is defective.

If difficulties are experienced with saline prepared with distilled water, use ordinary tap water. If still unsatisfactory, add 0.1 gram of magnesium sulphate to each 1000 c.c. as recommended by Kellogg.

SHEEP CORPUSCLES (INDICATOR ANTIGEN).—Sheep blood may be obtained at an abattoir or by bleeding a sheep from the external jugular vein. In a clean (but not necessarily sterile) quart-sized Mason jar, place 30 c.c. of a 10 per cent solution of sodium citrate in saline solution and 2 c.c. of formalin. At the abattoir have the jar almost filled with *fresh* blood (blood kept over in buckets is unsatisfactory), screw on the top, mix well with the citrate-formalin solution and keep in a refrigerator. Ordinarily it is fit for use over a period of two to three weeks at least, but as soon as the corpuscles become too fragile a fresh supply should be secured.

If preservation is not desired, glass beads alone may be placed in the jar and after filling it with blood thoroughly shaken for defibrination. Blood collected in this manner will keep at a low temperature (jar placed on a block of ice) for about a week. If beef blood is used, it may be collected by either method.

Filter a small quantity of blood through cotton into a graduated centrifuge tube. Allow twice as much blood as the amount of cells required. Add 2 or 3 volumes of saline solution. Centrifuge at moderate speed until all the corpuscles are thrown down.

Remove the supernatant fluid with a capillary pipet or by suction (Fig. 272). Add 3 or 4 volumes of salt solution; mix by inverting and centrifuge again for the same length of time.

Repeat the process for a third time but centrifuge twice as long as in the first washing in order to pack the cells evenly and firmly.

Cells should be washed until the supernatant fluid is almost colorless. Three washings are usually sufficient. (If more than four washings are necessary, the cells are too fragile for use.)

Read the volume of cells in the centrifuge tube, carefully remove the supernatant fluid and prepare a 2 per cent suspension by washing the corpuscles into a flask with 49 volumes of saline solution. *Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when not in use.*

ANTISHEEP HEMOLYSIN.—Give a rabbit five or six intravenous injections
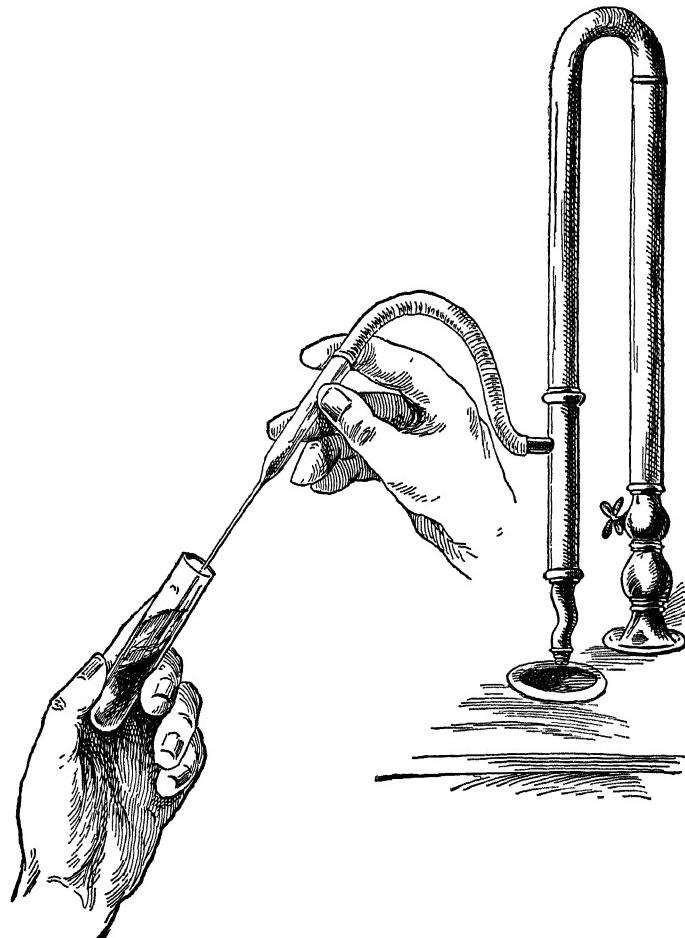
FIG 272—SUCTION PUMP.

(From Kolmer, *Infection, Immunity and Biologic Therapy,* W B. Saunders Co)

of 5 c.c. of a 10 per cent suspension of washed sheep corpuscles every five days (see Chapter II).   Bleed the rabbit seven to nine days after the last injection if a preliminary titration gives a unit of 0.5 c.c of 1 : 4000 or higher. Separate the serum and preserve with an equal part of best grade neutral glycerin.

COMPLEMENT.—The pooled sera of at least three healthy guinea-pigs should be used. Select large, well-nourished animals that have not been fed for twelve hours; avoid pregnant animals.

Anesthetize the pig lightly with ether. Sever the large blood vessels on both sides of the neck, being careful not to cut the esophagus. Collect the blood in a centrifuge tube by means of a large funnel. Place the blood in an incubator at 38° C. for one hour; break up the clot and centrifuge. Separate the clear serum. Keep in the refrigerator when not in use.

If but a small amount of serum is required, 4 or 5 c.c. of blood may be obtained from each pig by bleeding from the heart with a 5 c.c. syringe fitted with a short needle, gauge 20.

Complement serum may be preserved for several weeks by adding 0 3 gram of C.P. sodium chloride to each c.c. of serum. Keep in a dark glass bottle at or near the freezing point. To prepare for use, dilute 1 c.c. of serum with 29 c.c. of distilled water. This gives a 1 : 30 dilution in 1 per cent salt solution. As preserved complement loses first in fixability by syphilis antigen and antibody, it should not be kept for more than three or four weeks.

ANTIGEN.—A cholesterolized and lecithinized alcoholic extract of heart muscle is employed. Bacto-Beef Heart, prepared by the Digestive Ferments Company of Detroit, is recommended.

1. Place 25 grams of powder and 200 c.c. of ether in a tightly stoppered bottle. Extract at room temperature for five days, shaking occasionally each day. This extraction may also be made with 100 c c. of ether in a Soxhlet condenser for eighteen hours.

2. Filter off the extract and keep in a tightly stoppered bottle. This is the primary ether extract. Dry the powder by spreading on a glass plate. Return to bottle and extract with 200 c c. of 95 per cent alcohol in the incubator for four days, shaking occasionally.

3. Filter off the alcoholic extract, place in a shallow dish and evaporate to dryness by fanning, or in the incubator. Dissolve the residue in 30 to 50 c c. of ether. Cover and let stand for an hour or two. Pour off the clear ether extract and to it add the primary ether extract. Concentrate the mixture by fanning until it is reduced to about one-fourth of its volume. Add 6 volumes of pure acetone. Cover and set aside for several hours to allow the precipitate to settle. Decant the supernatant acetone and place the sticky residue in a wide-mouthed bottle. Cover with acetone and keep for future use. This is the acetone insoluble lipoid portion.

4. To the muscle powder left after extraction with 95 per cent alcohol add 100 c.c. of absolute acetone-free ethyl alcohol. Extract in the incubator for six days, shaking occasionally, and if possible shake in a mechanical shaker for one day. Filter through fat-free paper.

5. To the acetone insoluble lipoids add 0.2 gram of pure cholesterol. Dissolve the mixture in 10 c.c. of pure ether; add the cloudy brownish mixture slowly to the filtered alcoholic extract and shake well.

6. Place the antigen in the incubator overnight; then keep at room temperature for one or two days, shaking occasionally. Filter through fat-free paper. Keep in a tightly stoppered brown glass bottle at room temperature or in the refrigerator.

Preparation of Sera.—1. All specimens are lined up and properly labeled.

2. To each is added *a drop of washed sheet corpuscle sediment for approximately each 2 c.c. of blood and serum* as gauged by inspection.

3. Each specimen is then thoroughly mixed with a *perfectly clean* glass rod (need not be necessarily sterile).

4. All are then placed in a *refrigerator for fifteen minutes* to enable the sheep corpuscles to absorb the hemolysins with none or but a minimum and harmless amount of hemolysis.

5. All specimens are now centrifuged with due care to have the centrifuge properly balanced by carefully counterbalancing each pair of centrifuge tube holders on a balance and placing the holders opposite to each other in the centrifuge. For nice adjustment of balance, small glass beads are convenient.

6. The sera are now separated into test tubes properly labeled. Some of the sera may be tinged with hemoglobin but this does no harm.

7. The tubes are now placed in a water bath at 55° C. for *fifteen to twenty minutes* when they are ready for testing.

Preparation of Spinal Fluids.—*These are usually tested without any preliminary preparation* as they do not contain enough natural antisheep hemolysin to require removal or enough complement to require inactivation by heating at 55° C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged. Otherwise no preparation is required as spinal fluids are tested as delivered without preliminary heating unless they are more than a week old, in which case they may be heated at 55° C. for fifteen minutes to remove thermolabile anticomplementary substances. As a general rule, however, spinal fluids are not anticomplementary.

Titration of Hemolysin and Complement.—These titrations must be made each time the complement-fixation test is conducted.

HEMOLYSIN TITRATION.—For the hemolysin titration prepare a stock dilution of 1 : 100 hemolysin as follows:

Glycerinized hemolysin..... .... 2.0 c.c.
Saline solution........... .... 94.0 c.c.
Phenol (5 per cent in saline sol.)............ 4.0 c.c.

This may be kept in the refrigerator for several weeks.

For titration, dilute to 1 : 1000 (0.5 c.c. of 1 : 1000+4.5 c.c. salt solution.

In a series of ten tubes, prepare dilutions as follows:

No. 1.   0.5 c.c. hemolysin (1 : 1000) = 1 : 1000
No. 2.   0.5 c.c. hemolysin (1 : 1000) + 0.5 c.c. saline solution = 1 : 2000
No. 3.   0.5 c.c. hemolysin (1 : 1000) + 1.0 c.c. saline solution = 1 : 3000
No. 4.   0.5 c.c. hemolysin (1 : 1000) + 1.5 c.c. saline solution = 1 : 4000
No. 5.   0.5 c.c. hemolysin (1 : 1000) + 2.0 c.c. saline solution = 1 : 5000
No. 6.   0.5 c.c. hemolysin (1 : 3000) + 0.5 c.c. saline solution = 1 : 6000
No. 7.   0.5 c.c. hemolysin (1 : 4000) + 0.5 c.c. saline solution = 1 : 8000
No. 8.   0.5 c.c. hemolysin (1 : 5000) + 0.5 c.c. saline solution = 1 : 10,000
No. 9.   0.5 c.c. hemolysin (1 : 6000) + 0.5 c.c. saline solution = 1 : 12,000
No. 10. 0.5 c.c. hemolysin (1 : 8000) + 0.5 c.c. saline solution = 1 : 16,000

Mix the contents of each tube thoroughly.

Prepare 1 : 30 dilution of complement for hemolysin and complement titrations by diluting 0.2 c.c. of complement serum with 5.8 c.c. salt solution.

Prepare a 2 per cent suspension of sheep corpuscles.

In a series of ten tubes set up the hemolysin titrations as follows:

Tube	Hemolysin, 0 5 c.c.	Complement, c.c. (1 : 30)	Saline Solution, c.c.	Corpuscles, c.c.
1	1 : 1000	0.3	1.7	0 5
2	1 : 2000	0 3	1.7	0.5
3	1 : 3000	0 3	1.7	0.5
4	1 : 4000	0 3	1.7	0.5
5	1 : 5000	0 3	1.7	0.5
6	1 : 6000	0.3	1.7	0 5
7	1 : 8000	0 3	1.7	0 5
8	1 : 10,000	0 3	1.7	0 5
9	1 : 12,000	0.3	1.7	0 5
10	1 : 16,000	0 3	1.7	0 5

Mix the contents of each tube and incubate in the water bath at 38° C. for one hour. Read the unit of hemolysin. *The unit is the highest dilution of hemolysin that gives complete hemolysis.*

Two units are used in the complement and antigen titrations and in the complement-fixation tests. Hemolysin is so diluted that 0.5 c.c. contains 2 units. For example, if the unit equals 0.5 c.c. of 1 : 6000, two units equal 0.5 c.c. of 1 : 3000. Dilute just enough hemolysin for the complement titration and the complement-fixation tests. Keep hemolysin and corpuscles in suspension in the refrigerator when not in use.

COMPLEMENT TITRATION.—For the complement titration use 1 : 30 dilution of complement prepared above. Dilute antigen so that 10 units are contained in 0.5 c.c. This dilution is made by placing the required amount of saline solution in a flask and adding antigen drop by drop. Shake the flask after

each addition of antigen. Prepare enough antigen dilution for the complement titration and the complement-fixation tests.

In a series of ten test tubes set up the complement titrations as follows:

Tube	Complement, c.c. (1 : 30)	Antigen, c.c. (10 units)	Saline Solution, c.c.	Water bath 38° C. for one hour	Hemolysin, c.c. (2 units)	Corpuscles, c.c. (2 per cent)	Water bath 38° C. for one hour
1	0.1	0.5	1.4		0.5	0.5	
2	0.15	0.5	1.4		0.5	0.5	
3	0.2	0.5	1.3		0.5	0.5	
4	0.25	0.5	1.3		0.5	0.5	
5	0.3	0.5	1.2		0.5	0.5	
6	0.35	0.5	1.2		0.5	0.5	
7	0.4	0.5	1.1		0.5	0.5	
8	0.45	0.5	1.1		0.5	0.5	
9	0.5	0.5	1.0		0.5	0.5	
10	None	None	2.5		None	0.5	

The smallest amount of complement giving complete hemolysis is the *exact* unit; the next higher tube is the *full* unit. *Two full units of complement (the dose) are used in the test and are so diluted that 1 c.c. contains the dose.* Example:

> Exact unit = 0.25 c.c. of 1 : 30 dilution
> Full unit = 0.30 c.c. of 1 : 30 dilution
> Two full units = 0.60 c.c. of 1 : 30 dilution

To calculate the dilution to use so that 1 c.c. contains the dose, divide 30 by the dose:

$$\frac{30}{0.6} = 50 \text{ or dilution 1 : 50 of serum}$$

Hyperactive complement serum may give a full unit in amounts less than 0.3 c.c. of 1 : 30 dilution, but when this occurs the full unit should be taken arbitrarily as 0.3 c.c., since the use of smaller amounts may yield unsatisfactory reactions.

Titration of Antigen.—The antigen is titrated for the hemolytic, anticomplementary and antigenic units.

For the hemolytic and anticomplementary titrations prepare the following dilutions:

> 0.5 c.c. antigen   +1.5 c.c. saline solution = 1 : 4
> 0.5 c.c. antigen   +2.0 c.c. saline solution = 1 : 5
> 0.5 c.c. antigen   +2.5 c.c. saline solution = 1 : 6
> 1.0 c.c. antigen (1 : 4) +1.0 c.c. saline solution = 1 : 8
> 1.5 c.c. antigen (1 : 5) +1.5 c.c. saline solution = 1 : 10
> 1.0 c.c. antigen (1 : 6) +1.0 c.c. saline solution = 1 : 12
> 1.0 c.c. antigen (1 : 8) +1.0 c.c. saline solution = 1 : 16
> 1.0 c.c. antigen (1 : 10) +1.0 c.c. saline solution = 1 : 20

Prepare a 1 : 10 dilution of *normal negative* human serum inactivated at 55° C. for fifteen to twenty minutes.

For the *hemolytic titration* set up a series of six tubes as follows:

Tube	Antigen	Normal Human Serum, c.c. (1 : 10) *	Saline Solution, c.c.	Shake tubes gently and place in refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath for 10 minutes	Corpuscles, c.c. (2 per cent)	Water bath 38° C. for one hour
1	0.5 c.c. of 1 : 4	0.5	1.5		0.5	
2	0.5 c.c. of 1 : 5	0.5	1.5		0 5	
3	0.5 c.c. of 1 : 6	0.5	1.5		0 5	
4	0.5 c.c. of 1 : 8	0.5	1.5		0 5	
5	0.5 c.c. of 1 : 10	0.5	1.5		0.5	
6	0.5 c.c. of 1 : 12	0.5	1.5		0.5	

* May be omitted, in which case add 2.0 c.c. of saline to each tube instead of 1.5 c.c.

After secondary incubation, place tubes in the refrigerator for several hours and read the results. *The hemolytic unit is the smallest amount of antigen just beginning to produce hemolysis.* As a rule this antigen is not hemolytic in dilutions higher than 1 : 4.

For the *anticomplementary titration* set up a series of ten tubes as follows:

Tube	Antigen, 0.5 c.c.	Normal Human Serum, c.c. (1 : 10)*	Comple-ment, c.c. (2 full units)	Shake tubes gently and place in refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath at 38° C. for 10 minutes	Hemolysin, c.c. (2 units)	Corpuscles, c.c. (2 per cent)	Shake and place in water bath at 38° C. for one hour
1	1 : 4	0.5	1.0		0.5	0.5	
2	1 : 5	0.5	1.0		0.5	0.5	
3	1 : 6	0.5	1.0		0.5	0.5	
4	1 : 8	0.5	1.0		0.5	0.5	
5	1 : 10	0.5	1.0		0.5	0.5	
6	1 : 12	0.5	1.0		0.5	0.5	
7	1 : 16	0.5	1.0		0.5	0.5	
8	1 : 20	0.5	1.0		0.5	0.5	
9	Saline solution, 0.5 c.c.	0.5	1.0		0.5	0.5	
10	Saline solution, 1.0 c.c.	None	1.0		0.5	0.5	

* May be omitted and 0.5 c.c. saline added instead.

Place tubes in the refrigerator for several hours and read. *The anticomplementary unit is the smallest amount of antigen producing some inhibition of hemolysis.* This antigen is usually anticomplementary in dilutions up to 1 : 6. Tube 9 is the serum control; tube 10 is the hemolytic system control. Both should show complete hemolysis.

For the *antigenic titration* proceed as follows: From the remainder of the

1 : 10 dilution of antigen prepared for the hemolytic and anticomplementary titrations make the following dilutions:

$$
\begin{aligned}
&\text{0.1 c.c. antigen (1 : 10)} \quad +2.9 \text{ c.c. saline solution} = 1 : 300 \\
&\text{0.1 c.c. antigen (1 : 10)} \quad +3.9 \text{ c.c. saline solution} = 1 : 400 \\
&\text{0.1 c.c. antigen (1 : 10)} \quad +4.9 \text{ c.c. saline solution} = 1 : 500 \\
&\text{1.0 c.c. antigen (1 : 300)} \quad +1.0 \text{ c.c. saline solution} = 1 : 600 \\
&\text{1.0 c.c. antigen (1 : 400)} \quad +1.0 \text{ c.c. saline solution} = 1 : 800 \\
&\text{1.0 c.c. antigen (1 : 500)} \quad +1.0 \text{ c.c. saline solution} = 1 : 1000 \\
&\text{1.0 c.c. antigen (1 : 600)} \quad +1.0 \text{ c.c. saline solution} = 1 : 1200 \\
&\text{1.0 c.c. antigen (1 : 800)} \quad +1.0 \text{ c.c. saline solution} = 1 : 1600 \\
&\text{1.0 c.c. antigen (1 : 1000)} +1.0 \text{ c.c. saline solution} = 1 : 2000 \\
&\text{1.0 c.c. antigen (1 : 1200)} +1.0 \text{ c.c. saline solution} = 1 : 2400 \\
&\text{1.0 c.c. antigen (1 : 1600)} +1.0 \text{ c.c. saline solution} = 1 : 3200 \\
&\text{1.0 c.c. antigen (1 : 2000)} +1.0 \text{ c.c. saline solution} = 1 : 4000
\end{aligned}
$$

Prepare a 1 : 10 dilution of a mixture of four or more strongly positive syphilitic sera heated at 55° C. for fifteen to twenty minutes. Set up the titration as follows:

Tube	Antigen, 0 5 c.c.	Positive Serum, c.c. (1 : 10)	Comple-ment, c.c. (2 full units)	Shake tubes gently and place in refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath at 38° C. for 10 minutes	Hemolysin, c.c.	Corpuscles, c.c. (2 per cent)	Shake tubes and place in water bath at 38° C. for one hour
1	1 : 800	0 5	1		0 5	0 5	
2	1 : 1000	0 5	1		0 5	0 5	
3	1 : 1200	0.5	1		0 5	0.5	
4	1 : 1600	0 5	1		0.5	0 5	
5	1 : 2000	0.5	1		0 5	0.5	
6	1 : 2400	0 5	1		0.5	0 5	
7	1 : 3200	0 5	1		0 5	0 5	
8	1 : 4000	0.5	1		0 5	0 5	
9	Saline solution, 0 5 c.c.	0.5	1		0 5	0.5	
10	Saline solution, 1 0 c.c.	None	1		0 5	0 5	

Place tubes in refrigerator for several hours and read. *The antigenic unit is the smallest amount of antigen producing complete inhibition of hemolysis.* Tube 9 is the serum control; tube 10 is the hemolytic system control. Both should show complete hemolysis. As a general rule the unit is 0.5 c.c. of 1 : 2000 to 1 : 4000 dilutions.

In the complement-fixation tests 10 antigenic units of antigens are used. The antigen is so diluted that 0.5 c.c. contains the dose. For example:

$$
\begin{aligned}
&\text{Antigenic unit} = 0.5 \text{ c.c. of } 1 : 3200 \\
&\text{Dose (10 units)} = 0.5 \text{ c.c. of } 1 : 320
\end{aligned}
$$

As a general rule this dose of 10 units is thirty to sixty times less than the anticomplementary unit, which largely accounts for the high specificity of the reaction.

This antigen will keep for a year or longer with no change in antigenic or anticomplementary value.

Quantitative Complement-Fixation Test.—1. *For each serum:*

(*a*) Arrange six tubes and place in them the following amounts of saline solution, respectively: 1.2, 0.5, 0.5, 2.0, 0.5, and 0.5 c.c.

(*b*) To tube 1 add 0.3 c.c. of inactivated serum. Mix by drawing up in the pipet several times. Transfer 0.5 c.c. to tube 2 and 0.5 c.c. to tube 6.

(*c*) Mix tube 2 and transfer 0.5 c.c. to tube 3. Mix tube 3 and transfer 0.5 c.c. to tube 4. Mix tube 4 and transfer 0.5 c.c. to tube 5; discard 1.5 c.c. Mix tube 5 and discard 0.5 c.c.

(*d*) Tubes 1 to 5 now contain 0.5 c.c. each carrying 0.1, 0.05, 0.025, 0.005 and 0.0025 c.c. of serum. Tube 6 (serum control) contains 1 c.c. carrying 0.1 c.c. serum.

2. *For each spinal fluid:*

(*a*) Arrange six tubes and place 0.5 c.c. saline solution in tubes 2, 3, 4, 5 and 6.

(*b*) In tubes 1, 2 and 6 place 0.5 c.c. spinal fluid. Mix tube 2 and transfer 0.5 c.c. to tube 3. Mix tube 3 and transfer 0.5 c.c. to tube 4. Mix tube 4 and transfer 0.5 c.c. to tube 5. Mix tube 5 and discard 0.5 c.c.

(*c*) Tubes 1 to 5 now contain 0.5 c.c. carrying 0.5, 0.25, 0.125, 0.0625 and 0.03125 c.c. of spinal fluid. Tube 6 (control) contains 1 c.c. carrying 0.5 c.c. of spinal fluid.

3. To the first five tubes of each set of serum or spinal fluid add 0.5 c.c. of diluted antigen (carrying 10 antigenic units).

4. After an interval of five to thirty minutes add 1 c.c. complement (2 full units) to each tube.

5. Include the following controls:

Antigen control containing 0.5 c.c. diluted antigen, 0.5 c.c. saline solution and 1.0 c.c. diluted complement (2 full units).

Hemolytic system control containing 1 c.c. saline solution and 1 c.c. diluted complement (2 full units).

Corpuscle control containing 2.5 c.c. salt solution.

Positive and negative serum controls set up in dilutions as described above.

6. Mix the contents of each tube by gently shaking and place in the refrigerator at 6° to 8° C. for fifteen to eighteen hours.

7. Place tubes in the water bath at 38° C. for ten to fifteen minutes (not longer).

8. To all tubes except the corpuscle control, add 0.5 c.c. of hemolysin (carrying 2 units) and to all tubes add 0.5 c.c. of 2 per cent corpuscle suspension (shaken up).

9. Mix the contents of each tube by gently shaking and place in the water bath at 38° C. for one hour.

10. The following table shows the set-up for the quantitative complement-fixation test:

Tube	Patient's Serum in 0.5 c.c.	Antigen, c.c. (10 units)	Interval of 10 to 30 minutes at room temperature	Complement, c.c. (2 full units)	Primary incubation in refrigerator at 6° to 10 to 18 hours, followed by 8° C. for 15 to 18 hours, 15 minutes at 38° C.	Hemolysin, c.c. (2 units)	Corpuscles, c.c. (2 per cent)	Secondary incubation in water at 38° C. for one hour
1	0.1 c.c.	0.5		1.0		0.5	0.5	
2	0.05 c.c.	0.5		1.0		0.5	0.5	
3	0.025 c.c.	0.5		1.0		0.5	0.5	
4	0.005 c.c.	0.5		1.0		0.5	0.5	
5	0.0025 c.c.	0.5		1.0		0.5	0.5	
6	0.1 c.c. (control)	None		1.0		0.5	0.5	
7	Antigen control: 0.5 c.c. saline solution	0.5		1.0		0.5	0.5	
8	Hemolytic control: 1.0 c.c. saline solution	None		1.0		0.5	0.5	
9	Corpuscle control: 2.5 c.c. saline solution	None		None		None	0.5	

11. After the secondary incubation place the tubes in the refrigerator for several hours to permit the settling of nonhemolyzed corpuscles. Read the degree of inhibition of hemolysis and record for each tube as: − (complete hemolysis); + (25 per cent inhibition recorded as 1); + + (50 per cent inhibition recorded as 2): + + + (75 per cent inhibition recorded as 3); + + + + (100 per cent inhibition recorded as 4). All serum, antigen and hemolytic controls should show complete hemolysis. The corpuscle control should show no hemolysis.

12. Reactions may be interpreted as follows (See Plate X):

(a) *Very strongly positive:* partial or complete fixation of complement in the first four or five tubes of the test. Occasionally a serum will show less fixation of complement in the first tube carrying 0.1 c.c. serum than in the second tube carrying 0.05 c.c.; examples of a very strongly positive reaction are 4431 −; 44421; 1442 −.

(b) *Strongly positive:* partial or complete fixation in the first three tubes. Examples: 441 − −; 421 − −; 241 − −.

(c) *Moderately positive:* partial or complete fixation in the first two tubes. Examples: 43 − − −; 31 − − −; 12 − − −.

(d) *Weakly positive:* partial or complete fixation in only the first tube. Examples: 4 − − − −; 3 − − − −; 1 − − − −.

(e) *Negative:* complete hemolysis in all tubes, − − − −.

PLATE X
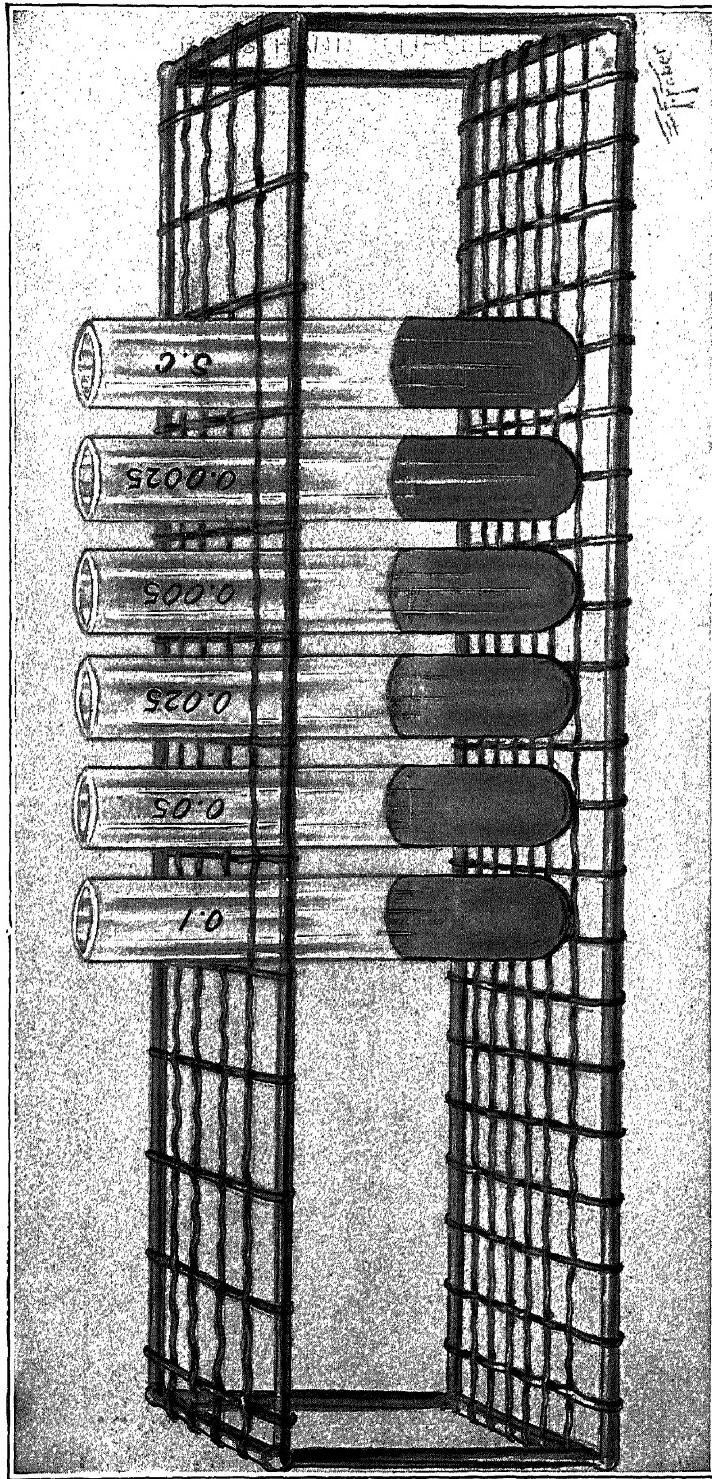
QUANTITATIVE REACTION.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

The method of recording and reporting a complement-fixation test by this method is as per the following example:

Quantitative reaction	= strongly positive (442 − −)
Serum 0.1 c c.	= + + + +
Serum 0.05 c.c.	= + + + +
Serum 0.025 c c.	= + +
Serum 0 005 c c.	= −
Serum 0 0025 c c.	= −
Serum 0.1 c.c (control)	= −

Quantitative reaction	= moderately positive (42 − − −)
Spinal fluid 0 5 c.c.	= + + + +
Spinal fluid 0 25 c c.	= + +
Spinal fluid 0 125 c c.	= −
Spinal fluid 0 0625 c.c.	= −
Spinal fluid 0 03125 c.c.	= −
Spinal fluid 0.5 (control)	= −

The Qualitative Complement-Fixation Test.—1. This test is conducted in exactly the same manner as described for the quantitative test except that two doses of serum (0.1 and 0.05 c.c. with 0.1 c.c. in the serum control) are employed. The 0.05 c.c. dose is included because sometimes the reaction is weaker and occasionally even negative with the 0.1 c.c. dose. With spinal fluid a single dose of 0.5 c.c. with 0.5 c.c. in the control is employed.

2. For each serum arrange three regulation tubes. Into No. 1 place 1.2 c.c. saline and 0.5 c.c. in Nos. 2 and 3.

3. Place 0.3 c.c. serum (heated 55° C. for fifteen minutes in No. 1; mix and transfer 0.5 c.c. to Nos. 2 and 3).

4. Mix No 2 and discard 0.5 c.c.

5. For each spinal fluid arrange two tubes and place 0.5 c c. (unheated) in each. The first tube receives antigen; the second does not and is the control.

6. Place 0 5 c.c. antigen (10 units) in Nos. 1 and 2 of each serum; also in control tube carrying 0.5 c c. saline (antigen control).

7. Allow tubes to stand five to thirty minutes. Then add two full units of complement (1.0 c.c.) to each; also to a control carrying 0.5 c.c. saline (hemolytic system control).

8. Put up corpuscle control: 2.5 c.c. saline and 0.5 c.c. of 2 per cent suspension.

9. Mix tubes gently and place in refrigerator at 6° to 8° C. for fifteen to eighteen hours. Keep hemolysin and corpuscle suspension in refrigerator.

10. Place tubes in water bath at 38° C. for ten to fifteen minutes (not longer).

11. To all tubes except corpuscle control add 2 units of hemolysin.

12. To all tubes except corpuscle control add 0.5 c.c. of 2 per cent corpuscle suspension (shaken up).

13. Mix and place in water bath for one hour. Refrigerator for hour or two.

14. The serum and hemolytic controls should be completely hemolyzed; the corpuscle control should show no hemolysis.

15. Read and record the serum tests by the $-$, $+(1)$, $++(2)$, $+++(3)$, and $++++(4)$ plan as follows:

$++++$ in either or both tubes = very strongly positive
$+++$ in either or both tubes = strongly positive
$++$ in either or both tubes = moderately positive
$+$ in either or both tubes = weakly positive
$-$ in both tubes   = negative

16. Read spinal fluid tests as follows:

$++++$ = very strongly positive
$+++$ = strongly positive
$++$ = moderately positive
$+$ = weakly positive
$-$ = negative

Modified Technic for Small Amounts of Serum and Spinal Fluid.—This modification enables one to conduct the quantitative test with but 0.1 c.c. of serum instead of the usual 0.3 c.c. and with 0.5 c.c. of spinal fluid instead of the usual 1.5 c.c. when larger amounts are not obtainable for the routine tests, particularly in the case of children. The technic is as follows:

1. *The hemolysin and complement are not titrated separately for this test but the same dilutions employed as in the routine test described above except that smaller amounts are employed as given below. The same antigen dilution is also used in a smaller dose.* In other words, the test is to be used in conjunction with the routine test for the examination of exceptionally small amounts of serum and spinal fluid which are almost surely to be submitted occasionally in routine work.

2. For each *serum* arrange six small test tubes and place saline solution, 1.4, 0.5, 0.5, 2 and 0.5 c.c., in the first five respectively.

3. Place 0.1 c.c. heated serum in No. 1; mix, transfer 0.5 c.c. to Nos. 2 and 6. Mix No. 2 and transfer 0.5 c.c. to No. 3; mix, transfer 0.5 c.c. to No. 4 and discard 1.5 c.c. Mix, transfer 0.5 c.c. to No. 5 and discard 0.5 c.c. The doses are one-third those of the routine test.

4. In testing *spinal fluid*, arrange six tubes and place saline solution as follows: 1 c.c. in No. 1 and 0.5 c.c. in Nos. 2, 3, 4 and 5. Into No. 1 place 0.5 c.c. of unheated fluid, mix and transfer 0.5 c.c. to Nos. 2 and 6. Mix No. 2 and transfer 0.5 c.c. to No. 3. Mix No. 3 and transfer 0.5 c.c. to No. 4. Mix No. 4 and transfer 0.5 c.c. to No. 5. Mix No. 5 and discard 0.5 c.c. The doses are one-third those of the routine test.

5. Add 0.2 c.c. of antigen dilution to the first five tubes of each set and 0.2 c.c. of saline solution to the last or serum control tube.

6. Prepare an *antigen control* with 0.2 c.c. antigen and 0.5 c.c. of saline solution.

7. Wait ten to thirty minutes and add 0.4 c.c. of the complement dilution for the routine tests to all tubes including the antigen control.

8. Prepare a *hemolytic system control* with 0.4 c.c. of complement dilution and 0.7 c.c. of saline solution.

9. Mix all tubes and place in refrigerator at 6° to 8° C. for fifteen to eighteen hours, followed by ten to fifteen minutes in a water bath at 38° C.

10. Add 0.2 c.c. of hemolysin dilution to all tubes.

11. Add 0.2 c.c. of the 2 per cent corpuscle suspension to all tubes.

12. Prepare a *corpuscle control* with 0.2 c.c. of suspension and 1.3 c.c. of saline solution.

13. Mix all tubes and place in a water bath at 38° C. for one hour. The total volume is 1.5 c.c. in all tubes.

14. Read the results at once or after waiting for an hour or two for the partial settling of corpuscles.

15. The readings are made and recorded in exactly the same manner as in the routine quantitative test described.

Analysis of Difficulties.—DEFECTIVE SALINE SOLUTION.—When trouble is experienced with the hemolytic system when first using these methods, it is likely that the saline solution is at fault. If it has been prepared with distilled water, try a saline prepared by dissolving 8.5 grams of chemically pure sodium chloride in 1000 c.c. of ordinary tap water; it is sometimes advantageous to add 0.1 gram of magnesium sulphate. If, however, trouble is experienced after a saline has been previously used with success it is unlikely to be the cause. Compressed tablets of salt should not be used.

DEFECTIVE COMPLEMENT.—The complement may be defective in hemolytic activity and this is especially true during the hot summer months when the diet of guinea-pigs may be responsible. As previously stated, a mixture of sera from *full grown*, healthy and *previously unused* pigs should be used. To be satisfactory the exact unit of complement should not be more than 0.3 c.c. of 1 : 30 dilution when titrated as described. If more than this the serum should not be used since the reactions are likely to be unsatisfactory because such complement is also very likely to be supersensitive to the anticomplementary effects of antigen alone and patient's sera alone.

DEFECTIVE HEMOLYSIN.—This is probably least likely to be a cause of trouble although usually first suspected, especially if the hemolysin has been previously used with success. The unit of antisheep hemolysin should be at least 0.5 c.c. of 1 : 4000 and sera of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory a good hemolysin is rarely responsible even when shipped over long distances.

DEFECTIVE CORPUSCLES.—When blood is obtained from an abattoir one is almost sure sooner or later to encounter the corpuscles of occasional animals possessing *increased resistance* to serum hemolysis. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood.

ANTICOMPLEMENTARY ANTIGEN.—Providing no mistakes have occurred in dilution and dosage, this is very rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is almost surely due to some component of the hemolytic system, especially the complement.

ANTICOMPLEMENTARY SERA.—Sera and spinal fluids may be found to be anticomplementary as shown by incomplete hemolysis of the serum controls. After experience has been gained some of these reactions may be safely read, but as a general rule it is safer and wiser to repeat the tests with fresh serum, especially in the case of those technicians lacking experience in complement-fixation work. It is infinitely better to repeat the tests than to run the slightest chances of error, especially the regrettable and almost unpardonable error of rendering a falsely positive report. Sometimes the majority of sera of a day's work show incomplete hemolysis of the serum controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests must be repeated and for this reason *the unused portions of all sera should be routinely kept in a refrigerator until the tests are completed in case repetitions are required.*

KOLMER COMPLEMENT-FIXATION TESTS FOR TUBERCULOSIS, GONORRHEA, TYPHOID FEVER, GLANDERS, CONTAGIOUS ABORTION AND OTHER BACTERIAL DISEASES

Since the technic of the various bacterial complement-fixation tests is almost exactly the same as for the syphilis reaction, it may be more briefly described. The methods of preparing the antigens are of most importance. The titration of antigen is exactly the same as in the syphilis reaction except that the dose employed in conducting the main tests is relatively much larger, being from one-third to one-fourth the anticomplementary unit. The primary incubation is the same except that after incubation at 6° to 8° C. for fifteen to eighteen hours, water bath incubation at 38° C. is for thirty minutes instead of the ten to fifteen minutes recommended for the syphilis test. A substitute primary incubation of two hours at 38° C. in a water bath may be used, especially in conducting the tuberculosis complement-fixation test.

Preparation of Bacterial Antigens.—TUBERCULOSIS.—1. Cultivate human tubercle bacilli in glycerin broth for about four weeks and autoclave the flasks at 10 pounds pressure for twenty minutes to kill the organisms.

2. Filter on several layers of good paper and wash the bacillary residue free of glycerin with sterile water.

3. Transfer the residue to a desiccator and dry over sulphuric acid.

4. Grind in a mortar under a hood for one half hour and keep in a tightly stoppered bottle at room temperature.

5. Place 1 gram of powder in a small Erlenmeyer flask fitted with a Liebig's condenser and electric heater and boil gently for one hour with 200 c.c. of ether. Discard the ether; dry the residue by placing the flask in an incubator, add 200 c.c. of acetone and boil for one hour. Discard the acetone, add 200 c.c. of absolute ethyl alcohol and boil for one hour. Discard the alcohol.

6. Dry the residue in the flask, add 190 c.c. of distilled water and boil for one hour. Add 2 grams sodium chloride to render isotonic and 10 c.c. of 5 per cent tricresol or phenol as a preservative. Store in a tightly stoppered bottle in a refrigerator for at least one week to ripen before titration.

GONOCOCCI, TYPHOID BACILLI, GLANDERS BACILLI, CONTAGIOUS ABORTION BACILLI, STREPTOCOCCI AND OTHER BACTERIA —*First Method.*—This method is especially adapted for preparing antigens of the gonococcus and other organisms difficult of cultivation on a large scale. Both it and the method that follows are based upon the principle of utilizing whole organisms.

1. Cultivate the organism on a suitable solid medium and wash off the growths with sufficient sterile distilled water to give a suspension containing approximately 2,000,000,000 per c.c. Or the organism may be cultivated in a suitable fluid medium, centrifugated and the residue suspended in sterile water to the same concentration.

2. Shake the suspension with glass beads for an hour to break up clumps.

3. Transfer to an Erlenmeyer flask fitted with a Liebig's condenser and electric heater and boil gently for two hours. Or the suspension may be boiled in an Arnold sterilizer for the same time, making up for any loss in volume with sterile water.

4. Add 1 gram of sodium chloride and 5 c.c. of 5 per cent tricresol or phenol to each 100 c.c.; stopper tightly and keep in a refrigerator for a week to ripen before titrating.

Second Method.—This method is particularly serviceable for preparing antigens of organisms readily secured in large amounts, as storing in dry powdered form provides a means for keeping indefinitely the base from which antigen may be made up in small amounts as required.

1. Cultivate the organism on a suitable solid medium and remove with a *minimum* amount of sterile saline solution or cultivate in a fluid medium and secure the organisms by centrifugation.

2. Dry the residue in a desiccator over sulphuric acid and grind for one half hour under a hood.

3. Store the powder in ampules or in a tightly stoppered bottle at room temperature.

4. For use place 1 gram in 190 c.c. of sterile distilled water and boil with a condenser for two hours; or boil in an Arnold sterilizer for the same time, making up for any loss in volume by adding distilled water.

5. Add 2 grams of sodium chloride and 10 c.c. of 5 per cent phenol or tricresol. Place in a tightly stoppered bottle in a refrigerator to ripen for a week before titrating.

Smaller amounts may be made up in the same proportions.

SALMONELLA PULLORUM (BACILLARY WHITE DIARRHEA OF CHICKENS).—*Method of Bushnell and Hudson.*—Cultivate *Salmonella pullorum* on agar. Wash off with saline solution. Shake vigorously for several minutes and filter through glass wool. Centrifuge for forty-five to sixty minutes at high speed. Discard the supernatant fluid. To each c.c. of sediment add 10 c.c. of ether. Mix well for four hours. Discard the ether. Add fresh ether and extract for two hours. Discard ether and dry residue in incubator. Suspend the residue in sufficient saline solution to give a turbidity equal to tube 3 of the McFarland nephelometer.

BRUCELLA ABORTUS (BOVINE INFECTIOUS ABORTION).—*Method of Boerner and Stubbs.*—Cultivate several strains on liver infusion or glycerin agar for four to seven days. Wash off with small amounts of sterile distilled water. Heat at 100° C. for three hours and place in refrigerator for ten days with occasional shakings. Then shake well and centrifuge at low speed for a short time to throw down a greater portion of the bacteria. The supernatant fluid, which should be still quite turbid, is pipetted off; add phenol to 0.5 per cent. Keep in refrigerator.

Titration of Bacterial Antigens.—The technic is exactly the same as described for titration of antigen for the syphilis reaction except that the dilutions are slightly different.

1. The following table gives directions for preparing the dilutions and it makes no difference whether antigen is added to saline or saline to antigen:

2.0 c.c. antigen (full strength)+2.0 c.c. saline solution = 1 : 2
1.0 c.c. antigen (full strength)+2.0 c.c. saline solution = 1 : 3
1.0 c.c. antigen (1 : 2)   +1.0 c.c. saline solution = 1 : 4
1.0 c.c. antigen (1 : 3)   +1.0 c.c. saline solution = 1 : 6
1.0 c.c. antigen (1 : 4)   +1.0 c.c. saline solution = 1 : 8
0.5 c.c. antigen (1 : 2)   +2.0 c.c. saline solution = 1 : 10
1.0 c.c. antigen (1 : 6)   +1.0 c.c. saline solution = 1 : 12
1.0 c.c. antigen (1 : 8)   +1.0 c.c. saline solution = 1 : 16
1.0 c.c. antigen (1 : 10)   +1.0 c.c. saline solution = 1 : 20
0.2 c.c. antigen (1 : 4)   +1.8 c.c. saline solution = 1 : 40
0.2 c.c. antigen (1 : 6)   +1.8 c.c. saline solution = 1 : 60
0.2 c.c. antigen (1 : 8)   +1.8 c.c. saline solution = 1 : 80
0.2 c.c. antigen (1 : 10)   +1.8 c.c. saline solution = 1 : 100
0.2 c.c. antigen (1 : 12)   +1.8 c.c. saline solution = 1 : 120
1.0 c.c. antigen (1 : 80)   +1.0 c.c. saline solution = 1 : 160
1.0 c.c. antigen (1 : 100)   +1.0 c.c. saline solution = 1 : 200
0.5 c.c. antigen (1 : 100)   +1.0 c.c. saline solution = 1 : 300
0.5 c.c. antigen (1 : 200)   +0.5 c.c. saline solution = 1 : 400

2. The following table shows the method of titrating for *hemolytic activity*, but since bacterial antigens prepared by the methods given above are rarely hemolytic, this titration may be safely omitted:

Tube	Antigen, 0.5 c.c.	Heated Human Serum,* c.c. (1 : 10)	Saline Solution, c.c.	6° to 8° C. for 15 to 18 hours, followed by 30 minutes in water bath	Corpuscles, c.c. (2 per cent)	Water bath for one hour	Readings
1	Full strength	0 5	1.5		0.5		Slight hemolysis†
2	1 : 2	0.5	1.5		0.5		No hemolysis
3	1 : 3	0 5	1.5		0.5		No hemolysis
4	1 : 4	0 5	1.5		0.5		No hemolysis
5	1 : 6	0 5	1.5		0.5		No hemolysis
6	1 : 8	0.5	1.5		0.5		No hemolysis

* May be omitted, in which case 2 c.c. saline are added to each tube instead of 1.5 c.c.
† Tubes containing large amounts of antigen should be centrifuged to determine any hemolysis.

3. The following table shows the method of titrating for *anticomplementary activity* and it is the most important of all; it must be conducted at frequent intervals with all antigens:

Tube	Antigen, 0.5 c.c.	Heated Human Serum,* c.c. (1 : 10)	Complement, c.c. (2 full units)	Refrigerator at 6° to 8° C. for 15 to 18 hours; or followed by water bath for 2 hours water bath only for 2 hours	Hemolysin, c.c., (2 units)	Corpuscles, c.c. (2 per cent)	Water bath for one hour	Readings
1	Full strength	0.5	1.0		0 5	0 5		No hemolysis
2	1 : 2	0 5	1.0		0.5	0 5		No hemolysis
3	1 : 3	0.5	1.0		0 5	0 5		Slight hemolysis
4	1 : 4	0 5	1.0		0 5	0.5		Slight hemolysis
5	1 : 6	0 5	1.0		0.5	0.5		Marked hemolysis †
6	1 : 8	0.5	1.0		0.5	0.5		Complete hemolysis
7	1 : 10	0 5	1.0		0.5	0.5		Complete hemolysis
8	1 : 12	0.5	1.0		0.5	0.5		Complete hemolysis
9	1 : 16	0.5	1.0		0.5	0.5		Complete hemolysis
10	1 : 20	0.5	1.0		0 5	0.5		Complete hemolysis
11	None; 0 5 c.c. saline solution	0 5	1.0		0 5	0.5		Complete hemolysis ‡
12	None; 2 5 c.c. saline solution	None	None		None	0.5		No hemolysis §

* May be omitted and 0.5 c.c. of saline added instead.
† Anticomplementary unit being largest amount of antigen giving slight inhibition of hemolysis.
‡ Hemolytic system and serum control.
§ Corpuscle control.

4. The following table shows the method of titrating for *antigenic activity*, which is not essential but always advisable if a suitable positive serum is available:

Tube	Antigen, 0.5 c.c.	Heated Positive Serum,* c.c. (1 : 10)	Complement, c.c. (2 full units)	for 15 to 18 hours; or 30 minutes; Refrigerator at 6° to 8° C. for followed by water bath for 2 hours; water bath only for 2 hours	Hemolysin, c.c. (2 units)	Corpuscles, c.c. (2 per cent)	Water bath for one hour	Readings
1	1 : 10	0 5	1 0		0 5	0 5		++++
2	1 : 20	0 5	1 0		0 5	0.5		++++
3	1 : 40	0 5	1.0		0 5	0 5		++++
4	1 : 60	0.5	1.0		0 5	0.5		++++ (†)
5	1 : 80	0.5	1.0		0 5	0.5		+++
6	1 : 100	0.5	1.0		0.5	0.5		+++
7	1 : 120	0.5	1 0		0 5	0.5		++
8	1 : 200	0.5	1.0		0 5	0.5		+
9	1 : 300	0.5	1.0		0.5	0.5		−
10	1 : 400	0.5	1.0		0 5	0.5		−
11	0 5 c.c. Saline solution	0.5	1.0		0.5	0.5		− ‡
12	1.0 c.c. Saline solution	None	1 0		0 5	0.5		− §

* 0.5 c.c of 1 : 10 dilution human positive serum heated at 56° C. for fifteen to twenty minutes; same if horse or guinea-pig immune sera are used; cattle sera 58° to 60° C. for thirty minutes; mule sera 62° C. for thirty minutes. But 0.5 c.c. of 1 : 50 if a rabbit immune serum (heated at 60° to 62° C. for thirty minutes) is being used.

† The antigenic unit being the smallest amount of antigen giving a + + + + reaction.

‡ Serum control giving complete hemolysis.

§ Hemolytic system control giving complete hemolysis.

The Dose of Antigen to Employ.—*A dose of bacterial antigen equivalent to one-third or one-fourth of the anticomplementary units is used.* For example, if the anticomplementary unit of an antigen happens to be 0.5 c.c. of 1 : 6 dilution, the dose may be 0.5 c.c. of 1 : 18, and the amount would probably carry 2 to 10 antigenic units as determined by the antigenic titration.

The antigenic titration is always advisable if a suitable positive serum is available, but if not, the anticomplementary titration alone may be made and the antigen used in an *arbitrary dose* equivalent to one-third or one-fourth of the anticomplementary unit.

Bacterial antigens keep very well in the refrigerator; it generally suffices to *titrate but once a month,* although one may titrate for anticomplementary activity each time tests are conducted by using the two-hour water bath incubation.

Quantitative and Qualitative Complement-Fixation Tests.—The technic of these is exactly as described above for the syphilis tests except that the primary incubation may be either (*a*) fifteen to eighteen hours in the refrigerator at 6° to 8° C. followed by *one half* hour in a water bath at 38° C. or (*b*) two hours in a water bath. The latter is particularly recommended for the tuberculosis complement-fixation test.

It is advisable to include positive and negative controls, especially the former. The negative controls should be of human sera in the tuberculosis,

gonococcus, typhoid and such tests; the positive controls should be of human sera when available but otherwise immune sera of the lower animals may be used.

The readings should be made ten minutes after complete hemolysis of the antigen control in the case of those sera showing complete hemolysis of the serum controls; these give the most sensitive readings. Otherwise the readings should be made *immediately* after the secondary incubation of one hour, providing the antigen, serum, hemolytic system and negative serum controls show complete hemolysis.

In conducting the tuberculosis complement-fixation test, the Wassermann test should be always conducted at the same time because syphilis antibody may give a positive reaction with tuberculosis antigen in the absence of tuberculosis. When the Wassermann reaction is strongly positive the tuberculosis test is also quite apt to yield a positive reaction and should be reported upon with great caution. This is not true, however, in the case of the gonococcus, typhoid and other bacterial complement-fixation tests.

KOLMER COMPLEMENT-FIXATION TESTS FOR TRYPANOSOMIASIS AND ECHINOCOCCUS DISEASE

Methods for preparing the various antigens are given below. The methods for titrating the hemolytic, anticomplementary and antigenic activities are exactly as given for the titration of bacterial antigens, the hemolytic system and general technic being exactly as described for syphilis.

Each antigen is employed in a dose equivalent to one-third of its anticomplementary unit since the large amounts yield the most sensitive reactions, prezone reactions being quite uncommon. Therefore the anticomplementary unit of each antigen must be known on the basis of preliminary titrations.

In conducting the main tests, either the quantitative or qualitative technic may be employed with a primary incubation of fifteen to eighteen hours in a refrigerator at 6° to 8° C. followed by one half hour in a water bath at 38° C., or the water bath only may be employed for two hours; the former has yielded the more sensitive and satisfactory results.

In conducting the echinococcus and other tests with human sera, it is advisable (indeed necessary) to conduct a Wassermann test at the same time because all of these antigens are capable of yielding cross complement-fixation reactions with sera containing large amounts of syphilis antibody. Whenever a serum gives a positive Wassermann reaction, the results of positive echinococcus or other reactions should be interpreted with due care.

When rabbit immune sera are used for positive controls, the dose should not be more than 0.5 c.c. of 1:50 dilution (0.01 c.c. serum) in order to avoid the nonspecific reactions sometimes yielded by normal rabbit serum with these antigens.

Antigens of Trypanosomes.—As a general rule these are prepared of

Trypanosoma equiperdum for complement-fixation tests with the sera of horses for dourine.

White rats are inoculated and as soon as the tail blood shows the presence of a heavy infection, antigens are prepared by securing the organisms from the blood by the method of Reynolds and Schoening as follows: "Blood of infected rats is collected in a 1 per cent sodium citrate solution in physiological salt solution in order to prevent coagulation. When all the blood has been collected, the solution is filtered through cheesecloth to remove clots, fibrin, etc., poured into tubes, and centrifugalized for about twenty minutes at 2100 revolutions per minute. This precipitates all the corpuscles and most of the trypanosomes, leaving an upper stratum of blood serum and citrate solution containing some of the organisms. This fluid is drawn off and again centrifugalized in order to recover any of the protozoa which may be present. To the other tubes containing the mass of corpuscles intermixed with and superimposed by trypanosomes is added sufficient distilled water to produce complete hemolysis of the rat erythrocytes, a matter of about twenty minutes, which procedure is facilitated by agitation of the mixture in a flask. This also is centrifugalized but in this instance for about one half hour, upon the completion of which there is found at the bottom of the tubes a mass of trypanosomes with an admixture of stroma of the hemolyzed red cells, which latter, in quantity, has been found to be negligible. After discarding the supernatant fluid (hemoglobin-stained water) physiological salt solution is added and the material vigorously shaken until the mass of trypanosomes is disintegrated and evenly distributed throughout the solution. Centrifuging is again resorted to with similar results, the washed mass of trypanosomes being packed at the bottom of the tubes. The salt solution is poured off and an amount of preserving fluid (physiological salt solution and glycerin) equal to about twice the amount of trypanosomes added; the mixture is then agitated until a uniform suspension is acquired, when it is stored at a low temperature until used."

Echinococcus Antigen.—The fluid from echinococcus cysts has been usually employed preserved with 0.5 per cent phenol in a refrigerator. It would appear, however, that the scolices contain most of the antigenic principles and a better antigen may be prepared by grinding up the moist scolices with fine sand in a mortar and adding 9 volumes of the clear cyst fluid or saline solution to give an approximate 10 per cent extract of the scolices. Phenol or tricresol should be added to 0.25 per cent and the mixture extracted in an incubator at 37° C. for four days, filtered, and stored in a refrigerator.

Fairley recommends an alcoholic extract prepared by grinding the scolices with fine sand, adding 9 volumes of absolute ethyl alcohol to give a 10 per cent extract, and placing the mixture in an incubator for two days when it is filtered and stored for use.

KOLMER COMPLEMENT-FIXATION TESTS WITH RABBIT, DOG AND MULE SERA

The sera of some of the lower animals, notably of the rabbit, dog, and mule, sometimes yield nonspecific complement-fixation and precipitation reactions with the various antigens employed in the syphilis reactions. With various bacterial antigens the degree of fixation is even greater. *Therefore, in conducting complement-fixation reactions with the sera of these animals, the technic must be modified to avoid the possibility of these nonspecific reactions and yet sufficiently sensitive for the detection of specific antibody.* These ends are met by heating the sera at 62° C. instead of at 55° to 56° C. and by using more complement (first method) or smaller doses of serum (second method).

First Method.—*The technic is exactly the same as described for the testing of human sera* except:

1. The natural antisheep hemolysins are not removed from the sera.

2. The sera are heated in a water bath at 62° C. for thirty minutes.

3. The doses in *syphilis* tests are the same as employed in testing human sera or they may be 0.1, 0.05, 0.025, 0.0125, 0.006 and 0.1 c.c. (control) in the quantitative test. But in *bacterial* complement-fixation tests smaller amounts of serum should be used as:

> 0.025 c.c. (0.5 c.c. of 1 : 20)
> 0.012 c.c. (0.5 c.c. of 1 : 40)
> 0.006 c.c. (0.5 c.c. of 1 : 80)
> 0.003 c.c. (0.5 c.c. of 1 : 160), etc.
> 0.025 c.c. (0 5 c.c. of 1 : 20) control

The first two doses of either series are used in the qualitative test, *i.e.,* 0.1, 0.05 and 0.1 c.c. (control) with cholesterolized and lecithinized alcoholic extract of beef heart antigen in syphilis tests, or 0.025, 0.012 and 0.025 c.c. (control) in bacterial complement-fixation tests employing the antigen in a dose equivalent to *one-fourth* of its anticomplementary unit.

4. *Four full units of complement* are used instead of two and so diluted that this dose is contained in 1 c.c. Example:

> Exact unit = 0.25 c.c. of 1 : 30
> Full unit = 0.30 c.c. of 1 : 30
> Four full units = 1.2 c c. of 1 : 30

To calculate the dilution to use so that 1 c.c. contains the dose, divide 30 by the dose:

$$\frac{30}{1.2} = 25 \text{ or dilution } 1 : 25 \text{ in dose of } 1 \text{ c.c.}$$

Second Method.—*The technic is exactly the same as described for testing human sera* except:

1. The natural antisheep hemolysins are not removed from the sera.
2. The sera are heated in a water bath at 62° C. for thirty minutes.
3. The doses of serum in quantitative *syphilis* tests with the usual 10 units of antigen are:

<div align="center">

0.025 c.c. (0.5 c.c. of 1 : 20)

0.0125 c.c. (0.5 c.c. of 1 : 40)

0.006 c.c. (0.5 c.c. of 1 : 80)

0.003 c.c. (0.5 c.c. of 1 : 160)

0.0015 c.c. (0.5 c.c. of 1 : 320)

0.025 c.c. (0.5 c.c. of 1 : 20) control

</div>

The doses in quantitative *bacterial* tests with one-fourth of the anticomplementary unit of antigen are:

<div align="center">

0.0125 c.c. (0.5 c.c. of 1 : 40)

0.006 c.c. (0.5 c.c. of 1 : 80)

0.003 c.c. (0.5 c.c. of 1 : 160)

0.0015 c c. (0.5 c.c. of 1 : 320)

0.0008 c.c. (0.5 c.c. of 1 : 640)

0.0125 c.c. (0.5 c.c. of 1 : 40) control

</div>

In qualitative tests the first two doses of either series are employed along with the larger amount in the third tube or serum control.

Heating sera at 62° C. for thirty minutes does not destroy enough antibody in syphilitic rabbits or in rabbits and dogs immunized to various antigens to interfere with the sensitiveness of the reactions; nor does this degree of heating of mule sera interfere with the sensitiveness of the glanders complement-fixation test for which they are usually submitted.

COMPLEMENT-FIXATION TESTS WITH ANTICOMPLEMENTARY SERA, SPINAL FLUIDS, EXUDATES AND TRANSUDATES

As is well known, sera, spinal fluids, exudates and transudates (chancre secretions, pleural, pericardial, and peritoneal exudates and transudates) may become anticomplementary or capable in themselves of fixing or inactivating complement in the *absence* of antigens; this is especially true of old and contaminated sera, chancre and pleural exudates, by reason of the presence of bacteria. In routine complement-fixation tests the phenomenon is detected by the occurrence of incomplete hemolysis in the serum controls. Since any serum, spinal fluid, etc., may contain anticomplementary substances, one should never assume that they are absent but always and invariably include the serum controls in every complement-fixation test.

First Method.—It is possible to read safely some anticomplementary reactions *when the serum controls show only slight interference with hemolysis.* The following are examples in which *negative* reports were correctly rendered:

$$
\begin{array}{l}
1 - - - - 1 \\
2 - - - - 1 \\
2\ 1 - - - 2 \\
2 - - - - 2 \\
4 - - - - 2 \\
4 - - - - 3
\end{array}
$$

In other words, if the first tube shows the same or very nearly the same degree of inhibition of hemolysis as the serum control with none or but the slightest inhibition in the second tube it is practically certain that the serum contains no antibody and that a negative report may be rendered; indeed, the evidence of a negative reaction is even stronger under these conditions than if the serum were free of anticomplementary action because if even a trace of antibody were present, strong inhibition of hemolysis would occur in the second and even the third tubes of the quantitative test.

Positive reports are also possible of correct reading, especially by *experienced* serologists *whenever the serum control shows less than* $+++$ (3) reactions, although it is impossible to estimate the degree of positiveness (one reporting only a "positive" reaction):

$$
\begin{array}{l}
4\ 4\ 3 - - 2 \\
3\ 2 - - - 1 \\
4\ 1 - - - 1 \\
4\ 4\ 4\ 4 - 2
\end{array}
$$

It is true, however, that some risk of error may accompany reports of this kind and it is always advisable to repeat the tests; this is especially true in the case of inexperienced workers.

Whenever the serum control shows complete inhibition of hemolysis ($++++$ or 4), it is too risky to report, as, for example, in the following reactions:

$$
\begin{array}{l}
4\ 4\ 1 - - 4 \\
4\ 2 - - - 4 \\
4\ 4\ 3 - - 4
\end{array}
$$

Second Method.—Sera *deeply* stained with hemoglobin are always likely to be anticomplementary; likewise serum, spinal fluid, urine, milk, etc., containing large numbers of bacteria, and freshly collected chancre exudates.

When these are tested it is advisable to put up a control on each dose; this is readily accomplished in routine work by setting up two series of tubes in the usual manner; antigen is added to all tubes of the first row except No. 6 (serum control) but not to the second row, which serves as the controls. The

sixth tube of the second row could be omitted as it is a duplicate of No. 6 in the first row but it should be included in order not to disturb the usual routine. The following are examples of reactions employing the quantitative test:

First row: 4 4 4 1 − 4 ⎫ Syphilitic serum
Second row: 4 1 − − − 4 ⎭

First row: 4 4 4 1 − 3 ⎫ Syphilitic serum
Second row: 4 1 − − − 4 ⎭

First row: 4 4 1 − − 2 ⎫ Syphilitic spinal fluid
Second row: 3 − − − − 3 ⎭

First row: 4 2 − − − 4 ⎫ Nonsyphilitic serum
Second row: 4 1 − − − 4 ⎭

First row: 3 2 − − − 2 ⎫ Nonsyphilitic serum
Second row: 3 1 − − − 3 ⎭

First row: 4 4 2 − − 4 ⎫ Nonsyphilitic spinal fluid
Second row: 4 3 1 − − 4 ⎭

Whenever complement fixation in the first row is *markedly* greater than in the second row, as shown above with the two syphilitic sera and spinal fluid, one may safely report a positive reaction without attempting to express an opinion on the degree of positiveness; negative reactions may be reported when the differences between the first and second rows are very slight. But whenever the differences are less marked, it is unsafe and unwise to vouchsafe a positive report as, for example, in reactions of these kinds conducted with known sera allowed to become anticomplementary:

First row: 4 4 4 2 − 4 ⎫ Syphilitic serum
Second row: 4 4 1 − − 4 ⎭

First row: 3 1 − − − 2 ⎫ Syphilitic serum
Second row: 2 − − − − 2 ⎭

First row: 2 1 − − − 1 ⎫ Syphilitic serum
Second row: 1 − − − − 1 ⎭

First row: 4 4 4 2 1 4 ⎫ Syphilitic serum
Second row: 4 4 4 1 − 4 ⎭

First row: 4 3 2 − − 4 ⎫ Syphilitic spinal fluid
Second row: 4 2 − − − 4 ⎭

It is true that the first row carrying antigen almost always shows stronger reactions than the second row, but unless the differences are quite marked it is not safe to give a positive report because normal or negative but anticomplementary serum and spinal fluid always yield a greater degree of inhibition of hemolysis in the presence of antigen.

Sachs' Method.—Sachs has described a very useful method for testing anticomplementary sera. Sera very deeply tinged with hemoglobin do not

respond quite as well to this method. Rabbit and dog sera, and the sera of the lower animals may be treated in the same manner since it likewise removes the anticomplementary substances from the majority but not the substances responsible for the nonspecific complementary-fixation reactions sometimes yielded by the normal sera of rabbits, dogs, and mules. The method has not been applied to spinal fluids.

1. Heat o 5 c.c. of serum at 55° C. for fifteen minutes.

2. Add 4.1 c.c. of accurately titrated N/300 hydrochloric acid and mix.

3. After standing one half hour at room temperature, centrifuge thoroughly and discard the sediment.

4. To the supernatant fluid add 0.4 c.c. of 10 per cent sodium chloride solution. The acid is fixed by the precipitate of globulin; hence neutralization is unnecessary.

5. This gives a 1 : 10 dilution of original serum ready for testing.

6. For the *quantitative test* arrange the usual six tubes and place saline solution as follows:

> No. 1: none
> No. 2: none
> No. 3: 0.5 c.c.
> No. 4: 2.0 c.c.
> No. 5: 0.5 c.c.
> No. 6: none

7. In tubes Nos. 1 and 6 place 1 c.c. of prepared 1 : 10 serum; place 0.5 c.c. in No. 2; place 0.5 c.c. in No. 3, mix and transfer 0.5 c.c. to No. 4; mix, transfer 0.5 c.c. to No. 5 and discard 1.5 c.c.; mix No. 5 and discard 0.5 c.c. The doses are 0.1, 0.05, 0.025, 0.005 and 0.0025 c.c. with 0.1 c.c. in the serum control as in the usual test except that the total volume in tubes Nos. 1 and 6 is 0.5 c.c. more at the end of the test than in the usual method.

8. For the *qualitative* test it is only necessary to place 1, 0.5 and 1 c.c. respectively in three tubes, the third being the serum control.

9. The test is now completed in the usual manner.

KOLMER COMPLEMENT-FIXATION TESTS WITH CHANCRE EXUDATES

1. The fluid is heated in the usual manner at 55° C. for fifteen minutes. If the quantity is less than 0.8 c.c., sterile saline solution is now added to this amount.

2. In a series of two tubes are placed 0.3 and 0.1 c.c.; similar amounts are placed in a rear or second series of tubes. If a larger amount of fluid is available the doses may be larger in both series, as 0.5 and 0.2 c.c.

3. Saline is added to bring the total volume to 0.5 c.c. in all tubes of the first row and to 1 c.c. in the second row.

4. The usual 10 units of antigen in 0.5 c.c. are added to the tubes of the first row; none is added to the second row, which are the serum controls on each dose.

5. The usual 2 full units of complement are now added to all tubes, followed by the routine incubation of sixteen to eighteen hours at 6° to 8° C. and ten to fifteen minutes in a water bath, etc.

The following are examples of reactions:

First row: 4 2 }
Second row: 1 — } Positive

First row: 2 1 }
Second row: — — } Positive

First row: 3 1 }
Second row: 1 — } Positive

First row: 1 — }
Second row: — — } Doubtfully positive

First row: 2 — }
Second row: 2 — } Negative

First row: 1 — }
Second row: 1 — } Negative

KOLMER COMPLEMENT-FIXATION TESTS WITH URINE, MILK, TRANSUDATES AND EXUDATES

Transudates like pleural, pericardial, peritoneal and joint fluids are usually free of anticomplementary activity and may be tested in the same manner as serum. They should be heated at 55° C. for fifteen minutes. As a general rule, however, their antibody content is less than in serum and larger doses are sometimes required similar to those employed in testing spinal fluid (0.5, 0.25, 0.125 c.c., etc.).

Exudates like blister fluids and tuberculous pleural exudates are much more likely to be anticomplementary; likewise urine and milk.

Blister fluids may be tested as described above for chancre exudates since the amounts submitted are usually quite small.

Urine, milk and *exudates* (like pleural exudates for tuberculosis) should be freshly collected and kept at a low temperature until examined. Each should be heated at 55° C. for fifteen minutes and first tested for anticomplementary activity as follows:

1. Titrate hemolysin.

2. In a series of eight test tubes place 0.5 c.c. undiluted, 1: 2, 1: 3, 1: 4, 1: 6, 1: 8, 1: 12 and 1: 16 dilutions of the fluid to be tested. Titrate the complement and add 2 full units (1 c.c.); also 0.5 c.c. of saline solution.

3. Water bath at 37° C. for one hour.

4. Add 2 units of hemolysin and 0.5 c.c. of 2 per cent corpuscles.

5. Water bath one hour and read.

6. Include a hemolytic system and corpuscle control.

The smallest amount giving even slight interference of hemolysis is the anticomplementary unit and varies greatly with different specimens.

7. In setting up the complement-fixation tests, place one-fourth of the anticomplementary unit in a front and rear tube; for example, if this happens to be 0.5 c.c. of 1 : 4 dilution, use 0.5 c.c. of 1 : 16.

8. To the front tube add the usual dose of antigen and to the rear tube 0.5 c.c. of saline (control).

9. The complement is titrated in the presence of the antigen in the usual manner and 2 full units added to both tubes.

10. The primary incubation is the usual fifteen to eighteen hours at 6° to 8° followed by ten to fifteen minutes in a water bath, the test being finished in the usual way.

CHAPTER XXVIII

METHODS FOR CONDUCTING PRECIPITATION TESTS FOR SYPHILIS

KAHN PRECIPITATION TEST [1]

Standard Apparatus.—1. Antigen suspension vials: 5.5 centimeters in length and 15 centimeters in inside diameter (1.7 centimeters outside diameter).

2. Test tubes: 7.5 centimeters in length and 1 centimeter in inside diameter (1.2 centimeters outside diameter).

3. Pipets for measuring antigen suspension: (a) 1.5 or 1 c.c. graduated to 0.05 c.c., for measuring the 0.05 c.c. quantities of suspension; (b) 0.25 c.c. graduated to 0.0125 c.c. or 0.2 c.c. graduated to 0.001 c.c., for measuring the 0.025 and 0.0125 c.c. amounts of antigen suspension.

4. Pipets for measuring serum: 1.5 c.c. graduated to 0.05 c.c. or 1 c.c. graduated to 0.01 c.c.

5. Test tube racks: These racks are made of sheet copper and are 3 inches wide, 11½ inches long and 2¾ inches high. They are constructed of three shelves, upper and middle ones containing three rows of ten holes, each of approximately one-half inch in diameter. The holes of the center row are offset one-half inch. The bottom shelf serves as a support.

6. Shaking apparatus: There are several types of shaking apparatus available on the market. Most shakers are constructed to hold a maximum number of six racks (Fig. 273) and are adjustable to hold less than this number if it is desired. Other shakers are constructed with a capacity for holding less than six racks, for use in laboratories where small numbers of tests are performed. The standard speed ranges from 275 to 285 oscillations per minute, with a stroke of one and one-half inches. The speed should be checked from time to time to assure conformance to this requirement. It may be desirable to enclose the six-rack shaker in a wooden box to reduce the noise during the shaking period. Such a box should be lined with felt or some similar material which will absorb sound.

Preparation of Reagents.—SERUM.—Collect blood as for the complement-fixation test; separate the sera and heat at 56° C. for thirty minutes.

SALINE SOLUTION.—Dissolve 9 grams of chemically pure sodium chloride in 1000 c.c. of distilled water and filter.

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[1] For a more detailed description of the test, including the procedure with spinal fluid, consult *The Kahn Test*, Williams and Wilkins Company, Baltimore, 1928 ed.

ANTIGEN.—The antigen for the Kahn test is a specially prepared alcoholic extract (cholesterolized) of powdered beef heart from which the ether-soluble elements have been partially removed.

Powdered beef heart for preparing antigen is now obtainable on the market in the form of Bacto-Beef Heart, which gives highly uniform results, due undoubtedly to the fact that a large number of hearts are used in making a given lot. Because of this uniformity, and also because of the labor- and time-saving factors, it is advantageous to employ this product in the preparation of antigen.

1. Fifty grams of powdered beef heart are placed in a 500 c.c. Erlenmeyer flask. Two hundred c.c. of ether (anesthesia) are added and the flask is shaken at frequent intervals for ten minutes. At the end of this period the ether is filtered off. Gentle pressure is applied to the beef heart in the funnel by means of a spatula, to assure as complete removal of the ether
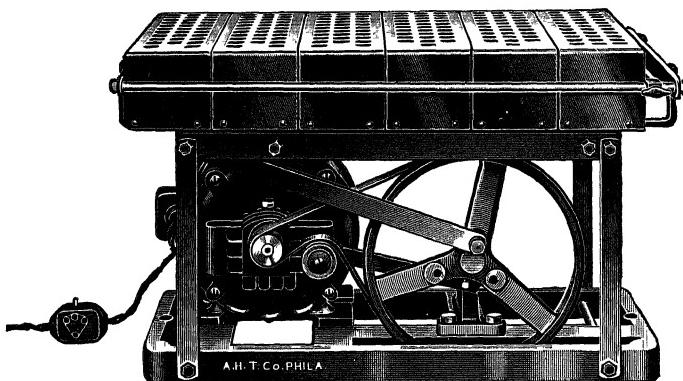
FIG. 273—KAHN SHAKING APPARATUS.

as possible. The filtration is completed when practically no drops of ether pass through the funnel as a result of pressure with the spatula.

2. The moist beef heart is transferred to the original flask. This may be done by first transferring the beef heart from the funnel to a sheet of white paper and breaking the material with a spatula into pieces small enough for the mouth of the flask. One hundred and fifty c.c. of ether are added to the flask, which is again shaken at frequent intervals during a ten-minute period. The ether is then filtered off as in the previous case.

3. The heart muscle is returned to the flask a third time and again covered with 150 c.c. of ether. The flask is shaken from time to time during a ten-minute period and filtration carried out as previously.

4. The moist powder is then transferred to the Erlenmeyer flask for the fourth and last ether extraction. One hundred and fifty c.c. of ether are added to the flask and after a ten-minute extraction period with frequent shaking, the final filtration of the ether is carried out. It is well to employ

fresh filter paper for each filtration, but care should be taken to minimize the loss of powdered muscle by scraping as much of the residue as possible from the paper into the extraction flask. When the moist heart muscle has been freed from ether as completely as in the earlier filtrations, it is spread upon a sheet of white paper or a clean glass plate and dried in the incubator at 37° C. for about ten minutes, or for a somewhat longer period at room temperature. When the material is dry and free from ether odor, it is ready for extraction with alcohol. The same Erlenmeyer flask in which the ether extraction was carried out may be employed for the alcohol extraction, provided the ether has been completely removed before the adding of the powdered muscle and alcohol.

5. After completing the ether extractions, the dried powder is weighed and transferred to a 500 c.c. Erlenmeyer flask. Five c.c. of 95 per cent alcohol are added per gram of powder, the flask is shaken for ten minutes, and extraction continued for three days at room temperature (about 21° C.). The flask is not shaken again during this extraction period except for a five-minute period just before filtration. The alcoholic extract after filtration is kept at room temperature in the dark as stock solution. All corks employed in connection with the preparation and storing of antigen are covered with high-grade thin tin foil.

6. A given amount of the alcoholic extract is measured into an Erlenmeyer flask and 6 milligrams of cholesterol are added per c.c. of extract. The flask is placed in warm water bath and rotated to hasten solution of the cholesterol. When the latter is entirely dissolved, the antigen is filtered and is ready for standardization.

Titration of Antigen.—1. Measure 0.6, 0.8, 1.0, 1.1, 1.2 c.c. respectively of physiological salt solution into five standard antigen suspension vials (5.5 centimeters length, 1.5 centimeters diameter).

2. Measure into each of five similar vials 1 c.c. of cholesterolized antigen.

3. Prepare five antigen suspensions by mixing the 1 c.c. quantities of antigen with the varying amounts of salt solution, in series. Empty the salt solution into the antigen and as rapidly as possible (without waiting to drain the tube) pour the mixture back and forth six times. Permit the mixture to stand for thirty minutes.

4. Test for the dispersability in salt solution of the lipid aggregates present in the antigen-salt solution suspensions after thoroughly mixing as follows:

(*a*) Set up five series of three standard tubes (employed in performing the regular Kahn test with serum, 7.5 centimeters length, 1 centimeter diameter).

(*b*) Pipet 0.05, 0.025, and 0.0125 c.c. quantities of each of the five antigen suspensions, in series, to the bottom of the tubes, using a 0.02 or 0.25 c.c. pipet marked in o oo1 or o o125 c c. amounts.

When measuring the antigen suspensions in series, it is advisable to begin with the suspension containing the largest amount of salt solution, and end

with the one containing the least amount of salt solution. This will avoid carrying nondispersable lipid aggregates from one suspension to the other.

(c) With a 1 c.c. pipet, add 0.15 c.c. salt solution to each of the fifteen tubes.

(d) Shake the rack of tubes for three minutes in a shaking apparatus at a speed of 275 to 285 oscillations per minute. If no such apparatus is available, rapid shaking by hand will approximate this speed.

(e) Add 1 c.c. salt solution to the tubes containing the 0.05 c.c. amounts of antigen suspension, and 0.5 c.c. to the remaining tubes. Observe whether fluids are opalescent or contain aggregates.

INTERPRETATION OF TITRATION RESULTS.—When each of the five antigen suspensions are thus tested for the dispersability of the aggregates by means of the three tube tests, it will usually be found that the antigen suspensions prepared by mixing 1 c.c. antigen and 0.6 or 0.8 c.c. salt solution contain aggregates which are not completely dispersed in additional salt solution, whereas the three remaining antigen suspensions, containing 1 c.c. amounts of antigen and 1, 1.1 and 1.2 c c. salt solution, respectively, contain aggregates which are completely dispersed in additional salt solution. The smallest amount of salt solution which, when added to 1 c.c. of antigen, produces aggregates capable of complete dispersion upon the addition of further salt solution, is the titer of the antigen. If the three-tube test indicates that 1 c.c. amounts of antigen mixed with either 1, 1.1 or 1.2 c.c. salt solution result in suspensions which are completely dispersable, then the titer of the antigen is 1+1. It should be added that antigen suspensions that are dispersed in additional salt solution are likewise dispersed in serum. If 1.2 c.c. salt solution added to 1 c.c. antigen results in a suspension containing particles which are not dispersable in additional salt solution, the titration is continued with volumes greater than 1.2 c.c. Rarely, one encounters an antigen the titer of which requires 1.5 c.c. salt solution or an even larger volume of salt solution per c.c. of antigen.

Determination of Sensitiveness of Antigen.—The sensitiveness of a new antigen is determined by comparing it with "standard Kahn antigen."

1. PREPARATION OF SYPHILITIC SERA FOR COMPARATIVE TESTS.—Ten sera are obtained, eight from syphilitic patients and two from nonsyphilitic individuals. Of the eight sera, at least six should give weakly positive reactions, and the remaining, strongly positive reactions. All sera are heated for thirty minutes at 56° C. before being tested. If the sera employed have been previously heated for thirty minutes, they should be reheated for ten minutes at the same temperature before use.

2. TESTING NEWLY PREPARED AND STANDARD ANTIGEN WITH SERA.— Antigen suspensions are prepared with both antigens in accordance with their respective titers. Both suspensions are permitted to stand for ten minutes and each is pipetted in 0.05, 0.025, and 0.0125 c.c. amounts for a series of ten Kahn tests. The sera are then added in 0.15 c.c. amounts. All the tests are

shaken for three minutes at 275 oscillations per minute and after adding the proper amounts of salt solution to each tube, the results with the two antigens are compared.

3. INTERPRETATION OF RESULTS.—If the results of the comparative tests with the two antigens are closely comparable, the new antigen probably possesses standard sensitiveness. To eliminate the possibilities of error, at least one additional series of comparative tests is carried out, and if the results are again comparable the newly prepared antigen may be considered as standard.

Correction of Antigen.—The sensitiveness of a newly prepared antigen may be greater or less than that of standard antigen. In either case it can readily be corrected to standard requirements. Two reagents are necessary for antigen correction: cholesterolized alcohol and sensitizing reagent.

PREPARATION OF CHOLESTEROLIZED ALCOHOL.—Cholesterolized alcohol is prepared similarly to cholesterolized antigen. Thus, for cholesterolizing 100 c.c. of 95 per cent alcohol the alcohol is added to 600 milligrams of cholesterol in a 250 c.c. Erlenmeyer flask or similar container. The cork to be employed should be covered with thin, high-grade tin foil. Rotate flask in a warm water bath until all cholesterol is dissolved. Filter to remove traces of foreign material. The solution is then ready for use.

PREPARATION OF SENSITIZING REAGENT.—1. The ether filtrate obtained in the preparation of antigen from 50 grams of heart muscle is refiltered to remove traces of powdered muscle, and is then evaporated with the aid of an electric fan.

2. When the volume has been reduced to about 10 c.c. or less, the concentrated ether extract is transferred to a small, weighed evaporating dish (capacity about 25 c.c.), the transfer being made complete by washing out the residue into the small dish with a little ether.

3. Evaporation is continued with the aid of the fan until the ether odor is no longer detectable.

4. At this stage there may separate from the dark brown lipid mass, a few c.c. of water. This water, which will be at the bottom of the evaporating dish, is removed by means of a capillary pipet. The lipid residue is brownish, semitransparent and viscous.

5. The evaporating dish is now reweighed, and the weight of the residue determined.

6. The residue is transferred to an Erlenmeyer flask (about 100 c.c. capacity). This is best accomplished with the aid of a small spatula.

7. A volume of absolute alcohol equivalent to 10 c.c. per gram of residue is added to the flask. A small amount of this alcohol is employed for rinsing the evaporating dish.

8. Extraction is allowed to take place for ten minutes at room temperature with frequent shaking of the flask. Comparatively little of the residue is soluble in alcohol, lipid masses being distributed throughout the mixture.

9. The mixture is placed in the ice box (4° to 9° C.) for three hours.

10. The mixture is filtered while cold and the flask containing the clear filtrate placed in the incubator at 37° C. for twenty-four hours.

11. The clear filtrate is permitted to stand for three days at room temperature. If a precipitate forms during this test period, the solution is refiltered.

12. The filtrate is cholesterolized with 6 milligrams of cholesterol per c.c. according to the usual technic.

13. The cholesterolized extract, known as "sensitizing reagent," is filtered and is then ready for use.

METHODS OF CORRECTION.—In order to understand the methods of correcting antigen to "standard" sensitiveness, it is necessary to recall that antigen sensitiveness (according to Kahn) is directly related to the concentration of lipids in the antigen. Only at a certain lipid concentration does an antigen give maximum sensitiveness, while excessive or deficient concentration reduces antigen sensitiveness. Furthermore, the degree of sensitiveness of standard Kahn antigen does not represent the maximum sensitiveness of which an antigen is capable, but instead represents a definitely chosen conservative degree of sensitiveness in conformity with specificity. Some newly prepared antigens will thus be more and some less sensitive than standard antigen, depending on their concentration of antigenic lipids.

Antigens More Sensitive than Standard Antigen.—When an antigen is more sensitive than standard antigen, it could be corrected either by concentrating the lipids of the antigen or by diluting the antigen with cholesterolized alcohol (since excessive concentration or dilution reduces antigen sensitiveness).

For simplicity, the method of choice is that of dilution. Technic: To a small amount of the oversensitive antigen, such as 10 c.c., is added 1.5 c.c. cholesterolized alcohol (15 per cent dilution). The diluted antigen is now tested against the standard antigen, using weakly positive sera. If comparable, the entire lot of new antigen is diluted with 15 per cent of cholesterolized alcohol. If not comparable, then if the antigen after 15 per cent dilution is still more sensitive than standard, a higher dilution, such as 25 per cent, is tried; if 15 per cent dilution reduced the sensitiveness below that of standard, a lesser dilution, such as 10 per cent, is tried.

The method of lipid concentration for reducing antigen sensitiveness is resorted to only when it is found that an antigen requires an excessive dilution (beyond 30 per cent) of cholesterolized alcohol to bring the sensitiveness to standard requirements. Technic: One and one-half c.c. of noncholesterolized antigen is placed in a small evaporating dish and evaporated to dryness by means of an electric fan. The lipid residue is taken up in 10 c.c. of the oversensitive antigen (15 per cent concentration). The concentrated antigen is now tested against the standard antigen as above. If the new antigen is still more sensitive than standard, a higher concentration, such as 25 per cent,

is tried; if less sensitive than standard, the concentration is reduced to per-haps 10 per cent.

Antigen Less Sensitive than Standard Antigen.—An antigen is less sensi-tive than standard when it is either too rich or too poor in lipids. This differentiation in the quantity of lipids can be determined during the antigen titration. An antigen having a titer greater than 1.0 c.c. salt solution per 1.0 c.c. antigen and below standard sensitiveness is of excessive lipid concentra-tion while an antigen having a titer of only 0.8 or 0.9 c.c. of salt solution per c.c. of antigen is of insufficient lipid concentration.

An antigen which is less sensitive than standard because of excessive lipid concentration is corrected by dilution with cholesterolized alcohol, employing the identical technic described above for correcting oversensitive antigens. An antigen less sensitive than standard due to insufficient lipid concentration is corrected by adding a small amount of sensitizing reagent, such as 1 per cent. Technic: To 10 c.c. of the less sensitive antigen is added 0.1 c.c. of sensitizing reagent. The modified antigen is now compared with standard antigen in the usual way. If still less sensitive than standard, the amount of sensitizing reagent is increased to 2 or more per cent; if more sensitive, the amount of reagent is reduced to 0.5 per cent.

Varying the Amount of Salt Solution in the Titer.—An antigen is most sensitive when mixed with salt solution according to its titer. If the titer of an antigen, let us say, is 1 + 1.1, then antigen sensitiveness is gradually reduced by employing antigen suspensions of 1 + 1.2, 1 + 1.3, etc. Therefore, an antigen having a titer of 1 + 1.1 and more sensitive than standard antigen could obvi-ously be brought down to standard requirements by employing titers of 1 + 1.3, 1 + 1.5 or even greater amounts of salt solution. Since most studies on the Kahn reaction have been carried out with antigens ranging from 1 + 1 to 1 + 1.3, Kahn does not recommend the use of larger amounts of salt solution in the titer and prefers reducing the sensitiveness of antigens to standard requirements by diluting them with cholesterolized alcohol.

Special Considerations —When standardizing antigen from a given lot of powdered beef heart, it will be found that the same methods of correction will be applicable to all other antigens prepared from the same lot provided the beef heart is kept in the ice box. If the method of correction, for example, is by 15 per cent dilution, with cholesterolized alcohol, then the additional antigens prepared from the same lot will require the same amount of dilution. Once standardized, an antigen will not undergo change, provided it is kept properly corked in the dark at room temperature.

Performance of the Test.—It is well to have the necessary equipment for the test ready before preparing the antigen suspension. Have racks set up, tubes numbered, sera heated and pipets ready for measuring the antigen suspension and serum. For measuring the 0.05 c.c. quantities of antigen sus-pension, a 1.0 or 1.5 c.c. pipet may be employed, graduated in 0.05 c.c. amounts. For measuring the 0.025 or 0.0125 c.c. quantities, a 0.2 or 0.25 c.c. pipet may

be employed which is graduated either in 0.01 or 0.0125 c.c. amounts. If the graduations on these pipets are not well defined, it is well to mark off the desired measurements with a wax pencil.

1. PREPARATION OF STANDARD ANTIGEN SUSPENSION.—Mix antigen with physiological salt solution according to required titer. Thus, if the titer is 1 c.c. antigen plus 1.1 c.c. salt solution, proceed as follows:

(*a*) Measure 1.1 c.c. salt solution into a standard antigen suspension vial.

(*b*) Measure 1 c.c. antigen into a similar vial.

(*c*) Pour the salt solution into the antigen, and as rapidly as possible (without waiting to drain the vial) pour the mixture back and forth six times to insure thorough mixing.

(*d*) Allow the antigen suspension to stand for ten minutes before using. The suspension should not be used after thirty minutes standing.

One may mix more than 1 c.c. of antigen with a proportionately larger amount of salt solution, but not less than 1 c.c. This amount when mixed with salt solution will be sufficient for about fifteen tests.

2. MEASURING ANTIGEN SUSPENSION.—After the antigen suspension has stood for ten minutes, shake it well (closing the mouth of the vial with the thumb) and measure 0.05, 0.025 and 0.0125 c.c. amounts for each serum, delivering the suspension to the bottom of the tubes. When employing the standard rack which contains thirty tubes, measure 0.05 c.c. amounts in the tubes of the first row; 0.025 c.c. amounts in the tubes of the second row and 0.0125 c.c. amounts in the tubes of the third row.

3. MEASURING SERUM.—The serum should be added as soon as possible after the antigen suspension has been pipetted, to avoid undue evaporation from the suspension. When examining large numbers of sera, it is well for one worker to measure the antigen suspension and for another to follow with the sera. Add 0.15 c.c. serum to each of the 0.05, 0.025 and 0.0125 c.c. amounts of antigen suspension, and shake the rack of tubes vigorously for about ten seconds (by hand) to insure thorough mixing of the ingredients. The rack may now be set aside until a given number of tests—up to about sixty—is ready for the regular three-minute shaking period.

4. CONTROLS FOR EACH SERIES OF TESTS.—(*a*) Antigen control. When pipetting antigen suspension for a series of tests, use the last set-up of 0.05, 0.025 and 0.0125 c.c. amounts as controls, adding to each tube 0.15 c.c. salt solution instead of serum. Include with the regular tests. All three tubes should show freedom from precipitation.

(*b*) Serum control. Examine each serum for particles which might give the appearance of a specific precipitate. It is essential to determine, particularly in the case of positive reactions, that the serum used in the test is entirely clear. In such reactions it is well to dilute the serum with salt solution to correspond approximately to the final dilution of serum when the tests are read. This dilution of the serum may render visible fine particles which are invisible in undiluted serum. Method: Dilute 0.1 c.c. serum with 0.3 c.c. salt

solution; shake tube for three minutes and examine for particles. If particles are present, the serum should be cleared by centrifugation and retested.

(c) Positive and negative controls. Include one or more sera which are known to give positive reactions and a similar number of sera giving negative reactions with each series of tests.

5. SHAKING.—The standard shaking period is three minutes. It is important not merely to agitate the rack of tubes but to see that the fluid within the tubes is vigorously agitated. When the tests are shaken by hand, one may shake each rack for three one-minute periods with short rest periods. When a shaking apparatus is employed, its speed should be not less than 275 oscillations and not more than 285 oscillations per minute, with a stroke of 1½ inch. When shaking by hand, this speed should be approximated.

6. ADDITION OF SALT SOLUTION.—After the serum-suspension mixtures have been shaken, add 1 c.c. salt solution to each tube of the first row of the rack (containing the 0.05 c.c. amounts of antigen suspension) and 0.5 c.c. salt solution to the remaining tubes. Shake sufficiently to mix ingredients.

7. READING OF RESULTS.—The results are read after the addition of the salt solution. Optimum reading conditions in each laboratory should be determined by trial.

When utilizing daylight for reading the tests, the following points will be found helpful:

(a) It is well to have but one source of light coming from a single window immediately in front of the observer. It will be found satisfactory to shade the upper and lower portions of the window, narrowing the source of light to a section several feet in height. Light from any other windows near the reader should be dimmed by lowering the window shades.

(b) When holding the rack in front of the exposed section of the window, the definitely positive and the negative reactions are readily differentiated without lifting the tubes from the rack.

(c) In the case of weak reactions, examine each tube individually, lifting it several inches above the eye level and slanting it until the fluid is spread into a thin layer. The precipitate will then become readily visible.

8. *Types of Reactions.*—The reactions are read on a plus-sign basis (Fig. 274).

(a) Four-plus reactions. In these reactions, definitely visible particles are suspended in a transparent or opalescent medium. The individual particles are readily visible by direct examination, without lifting the tubes from the rack.

(b) Three-plus reactions. In these reactions, the particles are also definitely visible, but are less clear-cut than in four-plus reactions. The particles are not always distinguishable until the tube is lifted from the rack and examined individually.

(c) Two-plus reactions. In these reactions, finer particles are suspended frequently in a somewhat turbid medium. The particles cannot be distinguished until the tube is examined individually, usually by slanting.

(*d*) One-plus reactions. In these, still finer particles are suspended in a somewhat turbid medium.

(*e*) Doubtful reactions. In these, extremely fine particles, just within the visible range, are suspended in a somewhat turbid medium.

(*f*) Negative reactions. In these, the medium is transparent and opalescent and free from visible particles. In the rack, negative reactions are readily distinguished from weakly positive reactions by the fact that the latter appear turbid.

9. INTERPRETATION OF RESULTS.—The results in the individual tubes are read on a plus-sign basis. The interpretation of the results is also on this basis.
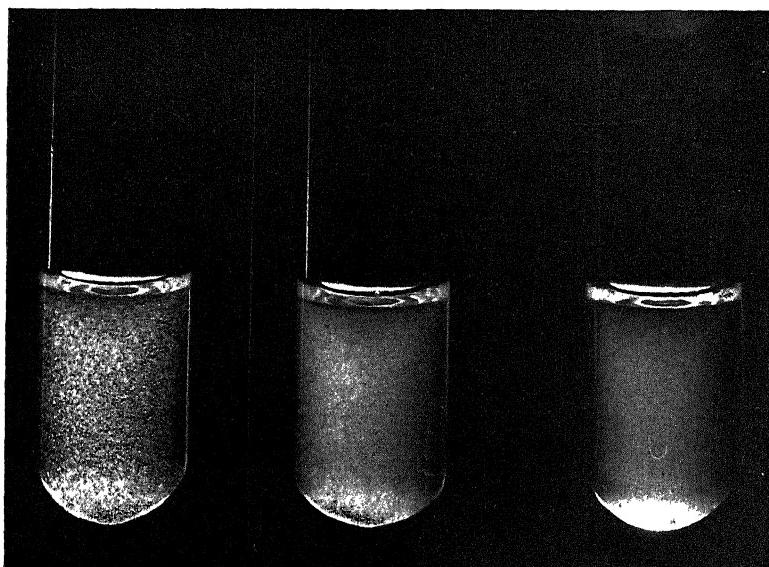
FIG 274—TYPES OF REACTIONS IN KAHN TEST.
(From *Kahn Test,* Waverly Press, Inc, Baltimore.

When each of the three tubes shows four-plus precipitation, the final result is interpreted as a four-plus reaction. When some of the tubes show weak or negative precipitation, such as negative in the first tube, two-plus in the second tube, and four-plus in the third tube, the final result is interpreted as a two-plus reaction. In other words, the final result is the average of the findings of the number of plus signs represented by the three tubes. If, after averaging, the final result contains a fraction of one-plus, then, if the fraction is one-third, it is disregarded; if the fraction is two-thirds, it is counted as an additional plus sign. Examples: $++$, $++++$, $++++$, is interpreted as a three-plus reaction, and $+$, $+++$, $++++$, is also interpreted as a three-plus reaction. When precipitation in an individual tube is read doubtful ($\pm$), it is not taken into consideration in averaging, but is counted as negative. Thus, $\pm$, $+$, $+++$ is

interpreted as a one-plus reaction (four-plus divided by three equals one-plus).
For reasons of conservatism, very weak reactions, *i.e.*, those in which precipita-
tion is limited to the third tube, no result is averaged unless this tube shows at
least a two-plus precipitate.   Thus −, −, ++ is interpreted as a doubtful (±)
reaction.   When the results in the three tubes are −, ±, +, the reaction is
interpreted as negative.   It might be added, however, that even the latter reac-
tions have been found to be highly specific for syphilis.   The following table
gives an outline of the test:

	Tube 1	Tube 2	Tube 3	Completion of Test
Serum: antigen suspension.	3 : 1	6 : 1	12 : 1	Tests are shaken three minutes, 1
Antigen suspension, c c.	0 05	0.025	0 0125	c.c. salt solution is added to first
Serum (heated at 56° C. for				tube and 0 5 c c. to other tubes
30 minutes), c.c. . .	0 15	0 15	0 15	and results are read.

TEMPERATURE FOR PERFORMING KAHN TEST.—It is essential that the
temperature of the room during the performance of the Kahn test be close to
21° C.   When the temperature is lower than 21° C., as is true in some labo-
ratories at certain seasons of the year, or when the test is performed in front
of an open window with cool air striking the antigen suspension and serum,
the precipitates in syphilitic sera will not be well formed and will be difficult
to read.   To avoid this difficulty the serum-antigen mixtures should be placed
in the water bath at 37° C. for about ten minutes before the three-minute
shaking period.   In laboratories where there are likely to be fluctuations in
temperature during different seasons of the year or where workers prefer to
perform the test in front of open windows, Kahn recommends the adoption
of a brief incubation period, such as ten minutes, at 37° C. during all the
seasons, for uniformity in results.

Performance of Quantitative Procedure.—1. SERUM DILUTIONS.—Pre-
pare a series of eight serum dilutions with physiological salt solution, so that
the ratio of the volume of diluted serum to the volume of serum before dilu-
tion ranges from 1 (undiluted serum) to 60 (1 part serum plus 59 parts salt
solution).   The following scheme is employed:

Dilution Number	*Dilution Ratio*		
(1)	1	=	undiluted serum
(2)	5	=	0.2 c.c. undiluted serum plus 0.8 c.c. salt solution
(3)	10	=	0.7 c.c. of (2) plus 0.7 c.c. salt solution
(4)	20	=	0.2 c.c. of (3) plus 0.2 c.c. salt solution
(5)	30	=	0.2 c.c. of (3) plus 0.4 c.c. salt solution
(6)	40	=	0.1 c.c. of (3) plus 0.3 c.c. salt solution
(7)	50	=	0.1 c.c. of (3) plus 0.4 c.c. salt solution
(8)	60	=	0.1 c.c. of (3) plus 0.5 c.c. salt solution

2. ANTIGEN SUSPENSION.—Prepare standard antigen suspension as for regular three-tube test and measure 0.01 c.c. amounts into eight standard test tubes.

3. MEASURING SERUM DILUTIONS.—Add 0.15 c.c. amounts of the eight serum dilutions, in order, beginning with the highest dilution (8), to the tubes containing antigen suspension. Mix ingredients by shaking for about ten seconds.

4. SHAKING.—Shake the mixtures of serum and antigen suspension by hand or in shaking apparatus for three minutes at 275 to 285 oscillations per minute.

5. ADDITION OF SALINE.—Add 0.5 c.c. salt solution to each tube and shake by hand for a few seconds to mix ingredients.

6. READING RESULTS.—After the salt solution has been added, the results are read. Record a definite precipitate ($++++$, $+++$ or $++$) as positive and a weak or negative precipitate ($+$, $\pm$ or $-$) as negative.

7. DETERMINATION OF REACTING (KAHN) UNITS.—If a serum gives a positive reaction only before dilution, it is considered as containing 4 units. The potency of any serum is determined according to the formula $S = 4D$, where S is the serum potency in terms of reacting units and D is the highest dilution ratio giving a positive reaction.

8. HIGHLY POTENT SERA.—If a serum gives a positive precipitation reaction with a dilution ratio of 60, examine still higher dilutions of serum with antigen suspension until a positive reaction is no longer obtained. Higher dilutions may be readily prepared by resorting to dilution (3), of which an excess is prepared. Thus, continuing the dilution numbers of the above outline, we would have:

Dilution Number	Dilution Ratio		
(9)	70	=	0.05 c.c. of (3) plus 0.3 c.c. salt solution
(10)	80	=	0.05 c.c. of (3) plus 0.35 c.c. salt solution
(11)	90	=	0.05 c.c. of (3) plus 0.4 c c. salt solution
(12)	100	=	0.05 c.c. of (3) plus o 45 c c salt solution

If necessary, still higher dilution ratios may be prepared, until a negative reaction is obtained. It is uncommon to find sera giving positive reactions in dilution ratios greater than 60 and rare to find a serum giving such reactions in dilution ratios greater than 100.

The Presumptive Procedure.—The presumptive procedure is a one-tube test and is more sensitive than the regular Kahn test by virtue of the fact that it utilizes a highly sensitive antigen known as "sensitized antigen." As is true in the case of standard Kahn antigen, sensitized antigen also possesses a uniform degree of sensitiveness. In preparing this antigen, standard antigen is used as a base and is brought to the required sensitiveness of sensitized antigen by means of sensitizing reagent in combination with cholesterolized

alcohol. Experience has shown that the addition of about 3 per cent sensitizing reagent to standard antigen followed by the addition of 10 or 20 per cent cholesterolized alcohol will considerably increase the sensitiveness of standard antigen. This fact is generally utilized in the standardization of sensitized antigen.

In preparing sensitized antigen using standard antigen as a base, the following steps are employed:

1. Ten sera are chosen which give weakly positive reactions in the three-tube Kahn test when using sensitized (instead of standard) antigen.

2. To 10 c.c. of standard antigen are added 0.1 c.c. of sensitizing reagent and 1.1 c.c. cholesterolized alcohol (1 per cent sensitizing reagent plus 10 per cent dilution with cholesterolized alcohol).

3. To a second 10 c.c. amount of standard antigen are added 0.2 c.c. of the sensitizing reagent and 1.1 c.c. cholesterolized alcohol (2 per cent sensitizing reagent plus 10 per cent dilution with cholesterolized alcohol).

4. These two modified antigens are titrated in the usual manner to determine the smallest amount of salt solution to add to 1 c.c. of antigen resulting in an antigen suspension, the aggregates of which will completely disperse in additional salt solution or in nonsyphilitic serum. In making this titration, antigen suspensions are prepared by mixing 1 c.c. amounts of antigen with 1.3, 1.5, 1.7, 1.9 and 2.1 c.c. quantities of salt solution, respectively. After these suspensions have stood for thirty minutes at room temperature, they are shaken and examined for the dispersability of the aggregates as follows: Each of the antigen suspensions is tested by employing the regular three-tube test except that after depositing the 0.05, 0.025 and 0.0125 amounts of the suspension in three tubes, 0.15 c.c. amounts of physiological salt solution instead of serum are added to each tube. After the usual three-minute shaking period, 1 c.c. salt solution is added to the tube containing the 0.05 c.c. amount of antigen suspension and 0.5 c.c. amounts of salt solution to the remaining tubes. The antigen suspension containing the smallest amount of salt solution and having aggregates which are completely dispersable in the additional salt solution, represents the titer of each of the modified antigens.

5. The two modified antigens at their titers are then compared in sensitiveness with a known standard sensitized antigen employing nine weakly positive sera and one negative serum. In these comparative examinations the regular three-tube test is employed. If one of the modified antigens is comparable in sensitiveness to the sensitized antigen, the comparative examination is repeated with ten additional weakly positive and ten negative sera, and if the results are again comparable, the new antigen is considered as standard sensitized antigen.

6. If neither one of the two modified antigens conforms to the requirements of standard sensitized antigen, other combinations of sensitizing reagent and cholesterolized alcohol are tried.

7. After the desired potency of sensitized antigen has been obtained, any amount of the antigen can be prepared by adding to standard antigen the determined amounts of sensitizing reagent plus cholesterolized alcohol.

PERFORMANCE.—1. Pipet 1 c.c. of standard sensitized antigen into an antigen suspension vial.

2. Pipet an amount of physiological salt solution, indicated by the titer, into a similar vial.

3. Pour the salt solution into the antigen and, as rapidly as possible, pour the mixture back and forth six times.

4. Allow the antigen suspension to stand ten minutes at room temperature before using.

5. Measure 0.025 c.c. of the thoroughly mixed antigen suspension into a standard tube (7.5 centimeters in length, 1 centimeter in diameter) with a 0.25 c.c. pipet marked in 0.025 c.c. amounts or with a 0.2 c.c. pipet marked in 0.001 c.c., delivering to the bottom of the tube.

6. Add 0.15 c.c. serum, after heating for thirty minutes at 56° C., with a 1 c.c. pipet graduated in 0.01 c.c., and mix the serum with the antigen suspension by shaking the rack vigorously by hand for about ten seconds.

7. Shake rack in the usual manner for three minutes (oscillation speed 275 to 285 per minute).

8. Add 0.5 c.c. physiological salt solution to the tube and examine for presence of precipitates.

9. The results are interpreted on a qualitative basis. Marked precipitation reactions, such as $++++$ or $+++$, are interpreted as positive; moderate precipitation reactions, such as $++$ or $+$, are interpreted as weakly positive, while very weak reactions such as $\pm$ are classed with the negatives.

10. Include antigen, serum, positive and negative controls as in the routine test.

VALUE OF PRESUMPTIVE PROCEDURE.—According to Kahn, the following are the most important purposes of this procedure:

1. As a technical check on the regular Kahn test. All sera giving positive reactions in the regular test should be positive with the presumptive procedure. A positive Kahn and negative presumptive indicate an error in technic.

2. As a check on weak Kahn reactions. A serum giving a doubtful ($\pm$) Kahn reaction should give a positive presumptive reaction; if negative, the doubtful reaction is most likely due to particles present in the serum.

3. As an aid in the diagnosis in certain cases of syphilis in which the regular Kahn test is negative.

4. Due to its high sensitiveness, the presumptive procedure, when negative, is a greater criterion for establishing absence of syphilis than the regular Kahn test.

KLINE MICROSCOPIC SLIDE PRECIPITATION TEST

Sera.—These are prepared as for the Wassermann test, care being exercised that they contain no red blood cells or foreign material. Before use, they are heated at 56° C. in a water bath for one-half hour.

Glassware.—Microscopic slides 2 by 3 inches as purchased are rubbed on both sides with Bon Ami paste (prepared by breaking up a cake of Bon Ami in a small quantity of hot water). As soon as the paste is dry (in about five minutes), it is completely removed from the slide with a soft muslin cloth. For convenience the slides covered with paste may be stuck to each other, allowed to dry and cleaned at any time. Upon the clean slides, twelve paraffin rings, each with an inside diameter of 14 millimeters, are mounted. After use the slides may be washed in hot water and prepared again as outlined above.

Instrument for Making Paraffin Rings.—This is essentially the instrument proposed by Green. A piece of soft iron wire (No. 28) 14 centimeters in length is wound twice tightly about a test tube about 15 millimeters in outside diameter, forming a double loop and leaving a double shaft about an inch in length. The two shafts are then twisted together to within a quarter of an inch of the free end. After removing the looped wire from the test tube, a piece of linen thread (No. 12) about a yard long is started from the free end of the shaft after being fastened there by a single twist of the two free ends. Three long turns are made reaching the loop which is then tightly wound with the thread; the winding is continued up the shaft to the free end where it is fastened between the two ends of the wire by twisting them. The loop is then bent at right angles to the shaft. It is then reshaped by working the loop against the bottom of the test tube mentioned above. The shaft is then inserted into the handle of a teasing needle or into a straight hemostatic forceps.

The paraffin rings are made by dipping the instrument into smoking paraffin (about 120° C.), draining quickly at one point and transferring the remainder to the glass slide.

Pipets.—The pipets needed for delivering the sera are the ordinary 1 c.c. pipets graduated in 0.01 c.c. The pipets for preparing the antigen emulsions are as follows: 1 c.c. graduated in 0.01 c.c. for distilled water; 2 c.c. graduated in 0.01 c.c. for cholesterin solution; 0.2 c.c. graduated in 0.001 c.c. for antigen, and 10 c.c. graduated in 0.1 c.c. for salt solution. The pipets for the antigen emulsions are Wright pipets made from glass tubing 6 to 10 millimeters in diameter with the tubes 3 to 5 millimeters in diameter, delivering a drop equal to about 0.008 c.c. (62 drops per one half c.c.).

Antigen.—The antigen is a liquid obtained from chilled absolute ethyl alcohol extract of beef heart muscle powder by precipitation in acetone at 50° to 37° C. It is prepared as follows:

1. Two hundred grams of dried heart powder (Difco) are placed in a 2 liter Erlenmeyer flask.

2. One liter of absolute ethyl alcohol (99+ per cent) is added.

3. After the flask is stoppered with a cork covered with tin foil, it is shaken

vigorously by hand at intervals for two hours. Better still, two wide-mouthed bottles (Difco bottles for one pound beef heart powder) each with 100 grams of beef heart powder and 500 c.c. of absolute ethyl alcohol (99+ per cent) are shaken vigorously in a machine for two hours. (This short extraction removed almost all of the desired antigenic substance in the powder.)

4. The extract is filtered into a liter cylinder through good grade filter paper of medium texture (Schleicher and Schull No. 597, 32 centimeters).

5. During filtration the mixture is stirred with a wooden tongue depressor and toward the end pressed with the cork until the powder is quite dry.

6. The extract (about 775 c.c.) is placed in the refrigerator at 8° to 10° C. for twenty-four hours.

7. During this time a fairly heavy white precipitate settles out. This is filtered off and the filtrate in a large evaporating dish is concentrated on a water bath at 45° to 50° C., determined by a thermometer bulb within the extract. During evaporation of the alcoholic extract an irregular festoon appears at the periphery. When the extract reaches the proper concentration the festoon disappears and the margin of the concentrated extract is sharp.

8. The extract is now poured quickly into 500 c.c. of chemically pure acetone (Coleman and Bell) at 50° C. in a large evaporating dish.

9. The dish is then placed in an air incubator at 37° C. for fifteen minutes after which the acetone is decanted, leaving a soft yellow brown wax adherent to the side of the dish.

10. The dish is then placed on a water bath or in an air incubator at 50° C. until the little acetone remaining has evaporated (about thirty minutes).

11. The wax is then worked together and placed in a glass-stoppered bottle. Eighty c.c. of absolute ethyl alcohol (99+ per cent), that have been kept in an air incubator at 50° to 56° C. for one half hour or longer, are added and after a few minutes shaking the bottle is placed in an air incubator at 50° C. and shaken gently after fifteen minutes and again after thirty minutes, when it is removed from the incubator and placed in the refrigerator at 8° to 10° C. for forty-five minutes.

12. The solution is then filtered and the filtrate is evaporated down at 45° to 50° C., resulting in a soft brown wax (antigen wax).

13. The wax is weighed and to each gram in a glass-stoppered bottle, 10 c.c. of absolute ethyl alcohol (99+ per cent) (at 50° to 56° C.) are added. After the bottle is shaken for a few minutes it is placed in an air incubator at 50° C. for thirty minutes, and then shaken a few minutes.

14. The slightly turbid solution is then placed at 8° to 10° C. for an hour and then filtered. The resultant clear filtrate is the antigen, and contains about 8.75 per cent of the alcohol-treated acetone-insoluble wax. (The average yield of antigen wax from a half pound of beef heart powder is 3.5 to 4 grams. The discarded acetone-soluble lipoidal residue (impurities) ordinarily weighs at least three times this amount).

The antigen is kept preferably in the incubator at 37° C. in a glass-stoppered bottle.

15. The 1 per cent cholesterin solution for the antigen emulsions is prepared as follows:

To 1 gram of cholesterin (Pfanstiehl C.P.) 100 c.c. absolute ethyl alcohol (99+ per cent) are added and the bottle placed in an incubator at 50° C. to 56° C. The bottle is shaken at fifteen-minute intervals to promote solution, which is complete in about forty-five minutes. This stock solution is kept in the incubator at 37° C. and is perfectly satisfactory for the preparation of the emulsions for as long as two months.

Antigen Emulsions.—1. Formula:

> Water (distilled) . ... 0.85 c.c.
> Cholesterin (C.P. Pfanstiehl in abs. ethyl
>   alc. 99+ per cent), 1 per cent . ... 1.00 c.c.
> Antigen   . .. . 0.10 c.c.
> Sodium chloride (C.P. or reagent, Merck)
>   0 85 per cent sol...... .... 2.45 c.c.

2. Into a 1-ounce bottle, 0.85 c.c. of distilled water is pipetted.

3. The bottle is held at an angle and the 1 c.c. of 1 per cent cholesterin in absolute ethyl alcohol (99+ per cent) is allowed to run along the side of the neck of the bottle.

4. The bottle is gently rotated from the neck for twenty seconds.

5. The bottle is held at an angle again and 0.1 c.c. of antigen is pipetted against the side of the neck from a 0.2 c.c. pipet (graduated in thousandths).

6. The bottle is then promptly stoppered with a cork and shaken vigorously (the fluid thrown from bottom to cork and back) for one minute.

7. Lastly, 2.45 c.c. of 0.85 per cent sodium chloride solution is allowed to run in quite rapidly, the bottle is stoppered again and shaken fairly vigorously for one minute.

8. The emulsion, when examined through the microscope, at a magnification of about 120 times, shows numerous very fine aggregates but no clumps whatever.

FOR DIAGNOSTIC TEST

Place 1 c.c. or more of the emulsion in a narrow test tube (12 millimeters inside diameter) in a water bath at 35° C. (beaker of water in usual laboratory minutes).

The emulsion as soon as heated is ready for use.

FOR EXCLUSION TEST

Place 2 c c. of the emulsion in a narrow test tube (12 millimeters inside diameter) in a water bath as 55° C. for fifteen minutes. Then pour into a 3 by 1 inch tube and centrifuge for fifteen minutes (about a thousand revolutions per minute). Decant the fluid and with the tube inverted dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 0.7 c.c. of 0.85 per cent sodium chloride solution. Transfer to a narrow tube for use.

These emulsions kept at room temperatures are satisfactory for use for forty-eight hours after preparation.

The diagnostic test when $++$, $+++$ or $++++$ may with very rare exceptions be relied upon as diagnostic of syphilis. The exclusion test, more sensitive than the diagnostic test, is not to be used for the diagnosis of syphilis, being of chief value when negative by indicating, in all but a small percentage of cases, the absence of syphilis.

The exclusion test is especially useful in testing prospective blood donors, in cases of syphilis under treatment and in cases presenting themselves with genital and mouth sores.

Method.—1. Into each of the thirty-six rings on three slides in a holder, 0.05 c.c. of the undiluted serum to be tested is delivered from a pipet (eighteen sera in duplicate). The tip of the pipet is placed in the center of the ring and the serum allowed to run out.

2. After all the sera are pipetted, one drop of the diagnostic test antigen emulsion (about 0.008 c.c.) is allowed to fall from a Wright pipet into one half of the sera. Into each of the other eighteen duplicate sera a similar drop of exclusion test antigen emulsion is allowed to fall.

3. After all the antigen is pipeted, the slides are rotated on a flat surface for four minutes and the results, magnified about 120 times, are read at once through the microscope (16 millimeter objective, 12 eyepiece) with the light cut down as for the study of urinary sediments.

4. Any spilling from a chamber makes the reaction therein unsatisfactory and the serum concerned should be retested.

Readings.—The contents of the chambers are examined in different portions and read negative if clumps are absent and $\pm$ to $++++$, depending upon the size of the clumps and upon the degree of the clumping.

SECTION V

CHEMICAL METHODS

CHAPTER XXIX

METHODS OF COLORIMETRY, NEPHELOMETRY AND SCOPOMETRY

Principles.—1. *Colorimetry* depends upon comparing and measuring the color of a fluid under examination with a similar solution of known strength, upon the principle that the depth of color is directly proportionate to the amount of the substance present.

2. *Nephelometry* depends upon measuring the density of precipitates and thus determining the amount of any substance which can be obtained in the form of a suitable suspension. It differs from colorimetry in that it uses reflected instead of transmitted light. The brightness of two fields is compared instead of their colors. It is adapted particularly for the determination of substances that are in very dilute and colloidal suspension, which do not precipitate appreciably in the time required for making readings. The method has been adapted to the determination of a large number of substances and is continually finding new applications. It is possible to determine very minute amounts of substances, entirely outside of the range of gravimetric methods of analysis, and hence may be used where the amount of material is very small. If properly carried out the limits of error are not greater and may be less than those of the colorimetric methods commonly employed.

3. *Scopometry* is closely related, but depends upon an extinction or vanishing point criterion as an optical measure of turbidity and color measuring the optical density of a sample until a point is reached at which the density of the sample plus the density added to it cause the image of a standard target to disappear. For adding density to the sample the Exton Junior Scopometer utilizes a continuously graded series of increasing densities in the form of an optical wedge having a predetermined standard slope and called neutral because it transmits equally all colors of the spectrum. By shifting the position of the wedge, the point on the wedge scale at which the target image seen through sample and wedge vanishes directly measures the concentration of the specimen. The results are usually easily read, of considerable accuracy and the methods particularly applicable to small amounts of test material.

COLORIMETRIC METHODS

Duboscq Method.—1. The general construction is shown in Figure 275. A modification by Hastings for determining hydrogen-ion concentration is shown in Figure 276.

2. The solutions to be compared are placed in glass-bottomed cups, which can be raised by means of rack and pinion until the lower ends of the clear glass plungers are immersed in the fluid, the excess of fluid rising between the plungers and the walls of the cups. By raising or lowering the cups the layer of fluid between the lower ends of the plungers and the bottom of the cups may be made of any desired thickness and the thickness of each is indicated by a scale placed in a convenient position. Beneath the cups is a mirror which reflects light up through the cups and the long axis of the plungers into a
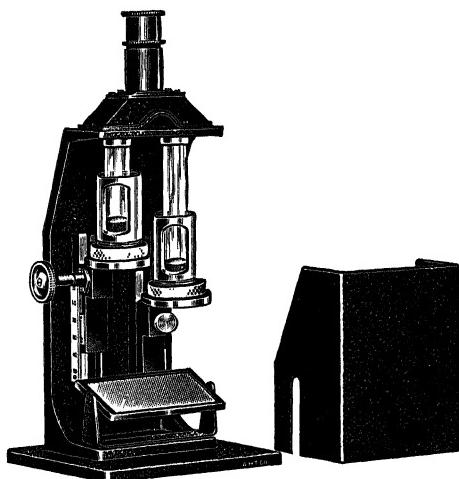
FIG 275—SMALL DUBOSCQ COLORIMETER
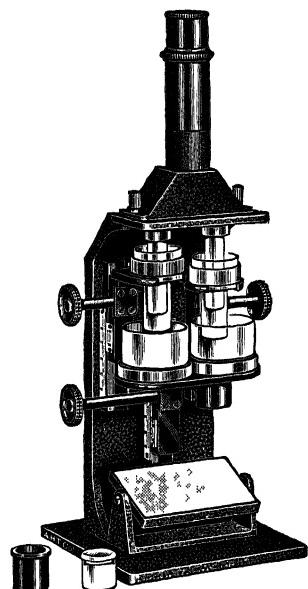
FIG 276—HASTING HYDROGEN ION COLORIMETER.

series of prisms. These reflect the light from the two cups into a single field which is viewed by an eye lens. Each lateral half of the field receives its light through one of the cups. The raising or lowering of the cups, by diminishing or increasing the thickness of the layer of fluid through which the light passes, diminishes or increases the depth of color of the corresponding half of the field.

3. If identical fluids are poured into both cups and the cups placed at the same height, then the two halves of the field should have exactly the same depth of color. If they do not exactly match, the scale, which is movable

upon most instruments, must be brought to accurate adjustment. In the absence of an adjustable scale a correction must be made in the height of the cup which is to receive the standard—customarily the left-hand cup. If, for example, with the right cup at 10 millimeters the reading of the left is 10 2 millimeters, then the left, which is to contain the standard solution, should be set at 10.2 when making the tests.

4. To use the colorimeter, focus the eyepiece and arrange the reflector so that the two halves of the field are equally illuminated. Fill one of the cups half full of the standard color solution and raise this cup until the layer of fluid between the bottom of the cup and the lower end of the plunger is of a convenient thickness. This will usually be 10 or 20 millimeters, as indicated by the scale. Place the unknown solution in the other cup, and move this cup up and down until the two halves of the field viewed by the eyepiece exactly match in color. Either daylight or artificial light which is filtered through daylight glass may be used. With daylight, readings are most accurate when the colorimeter is placed in front of a window, but far enough from it (6 to 10 feet) to avoid any strong light entering the eye. Artificial light is best used in a darkened room or a dark corner of the laboratory. Note the reading on the scale. The concentrations of the two solutions are inversely proportional to the respective readings when the colors match. This may be expressed in the formula:

$$\text{Strength of unknown} = \frac{\text{reading of standard} \times \text{strength of standard}}{\text{reading of unknown}}$$

If, for example, in the phenolsulphonephthalein test the 50 per cent standard be used, while the cup containing it is placed at 10 millimeters, and the unknown stands at 15 millimeters when the colors match then:

$$\text{Strength of unknown} = \frac{10 \times 50}{15} = 33\ 3 \text{ per cent}$$

Alternate Method —Bring both cups up as far as possible and read the error on the scale, if any, and add or subtract this error to the reading of respective cup.

Results are always most accurate when the unknown and the standard have nearly the same depth of color.

The Bock-Benedict Colorimeter (Fig. 277) employs the same general principle as the Duboscq. The standard solution is placed in a glass trough of standard thickness while the unknown is placed in a cup with plunger.

The Klett-Bio Colorimeter is a new colorimeter with several unique features, such as a built-in substage lamp and a revolving calculation table. The principle of operation is essentially the same as that of the Duboscq colorimeter.

Hellige Method.—1. This instrument (Fig. 278) is somewhat less accurate than the Duboscq type.

2. The solution under examination is placed in the box or trough, *B*, while the standard solution is placed in the wedge-shaped bottle, *C*, which can be moved up or down beside the trough. The front, *A*, is slipped into place, and the two solutions are viewed through the window, behind which is a double prism to bring the two colors close together. The wedge is moved up and down by means of the knurled head, *D*, until a point is reached where the two colors match. The figure on the scale which then stands opposite the pointer indicates the relation between the strengths of the two solutions. If the pointer stands at 40, then the unknown solution is 40 per cent as strong as the known standard; if at 70, it is 70 per cent as strong. Hermetically
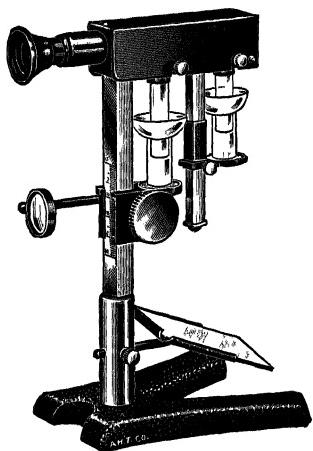
FIG 277—BOCK-BENEDICT
COLORIMETER.
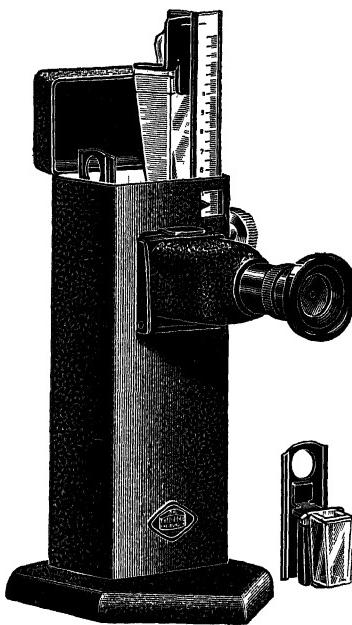
FIG 278—NEW HELLIGE UNIVER-
SAL WEDGE TYPE COLORIMETER

sealed standard wedges for most of the tests can be purchased with the instrument.

3. Before the instrument is used its accuracy should be tested. The following plan is simple (Todd and Sanford): Place the same colored solution—for example, a phenolsulphonephthalein solution—in both the wedge and the glass box, and rack the wedge up and down until the colors match The scale should then read 100. Now dilute the solution in the glass box exactly 1 : 10 (1 part of solution, 9 parts of water). This should give a reading of 10 when the colors match. Test the intermediate graduations of the scale in the same way. If the scale proves to be inaccurate, make a record of the figures to be added or subtracted.

NEPHELOMETRIC METHODS

1. The Duboscq Colorimeter has been adapted for nephelometric determinations by Kober, Bloor and others. The Bausch and Lomb attachment shown in Figure 279 is satisfactory, likewise those furnished by the International Instrument Company of Cambridge, Mass.

2. Directions for use usually accompany the instruments.

3. As stated by Hawk, "the amounts of precipitate in solutions examined nephelometrically are not exactly inversely proportioned to the readings of the
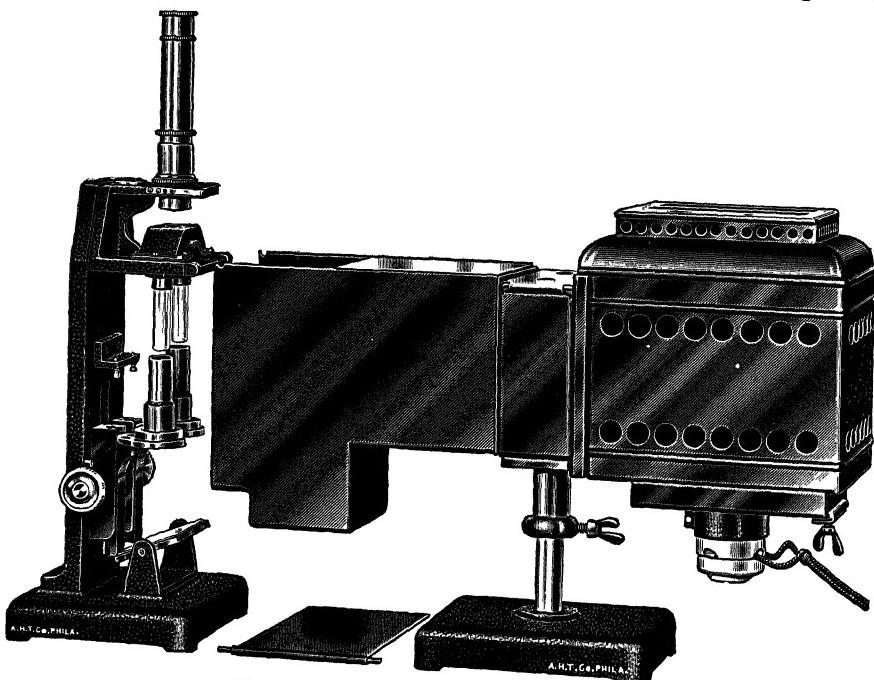
FIG. 279.—NEPHELOMETER ATTACHMENT.

scale. When the concentration of the unknown and of the standard are within 10 per cent of each other (or within about 20 per cent if the readings are made at depths as great as 50–60 mm.) accurate results may however be obtained directly. If the variations are greater than this a correction is necessary. Kober has proposed an equation to supply this correction and thus make possible very accurate work under conditions of moderate variations of concentration. The equation is as follows:

$$y = \frac{s}{x} - \frac{(1-x)sk}{x^2}$$

or

$$x = \frac{s + sk + \sqrt{(s+sk)^2 - 4sky}}{2y}$$

where y = height of unknown solution, on the left side of the instrument, when standard solution is kept on the right side at a definite height, s = height of standard solution on the left side and x = the ratio of the concentrations of the two solutions. $k = K/s$ where K = a constant, obtained by substitution of standardization values of s, y, and x. The instrument should be checked up for each series of analyses by reading the standard against itself and determining the potential height of the standard solution by reading the scale on the left side when the solution on the right side is kept at a definite height, and the two are matched."

SCOPOMETRIC METHODS

Scopometry is performed by three different instruments: the Scopometer, the Junior Scopometer, and the Electro-Scopometer.

The Junior Scopometer (Fig. 280) provides a handy and efficient means for making many different kinds of measurements without disturbing the sample, and is unique in offering extinction and photometric criteria as well as polarization measurements in the same instrument. It is supplied by Bausch and Lomb and the filters and wedge by the Eastman Kodak Company. Full directions accompany the instrument.

Visual acuity is pooled with all other variables that might affect measurements and in practice is almost wholly eliminated. The vanishing point criterion is affected much less than the older methods by color and flocculation differences. It also allows measuring the samples in the same tubes in which the tests are made, which makes for unexampled ease and rapidity of manipulation.

The exceptional feature is the entire freedom from all troubles and inaccuracies incidental to the preparation and control of comparison standards, which it dispenses with altogether. The measurements of cloudiness and color offered by this instrument make it possible to repeat measurements

FIG. 280.—THE EXTON JUNIOR SCOPOMETER.

(Bausch and Lomb Optical Co., Rochester, N. Y.)

of a sample without respect to the lapse of time, an advantage of practical value which may be particularly helpful to enthusiasts on permanent standards.

The use of light filters makes colorimetry with the Junior Scopometer often practicable in the presence of interfering colors and tends to extend greatly the range of measurement. Furthermore, by measuring the same sample with

selected filters, transmitting different frequencies and repeating such measurements, transformations of a fundamental chemical nature may be subjected to practically continuous observation, and it is not beyond conjecture that this may prove to be the most interesting, if not the most valuable, feature of colorimetry with the Junior Scopometer.

It might also be mentioned that the vanishing point criterion makes it possible to simplify technic to such an extent that determinations now regarded as too cumbersome for routine work may be brought within the realm of clinical pathology.

Although the Junior Scopometer is more accurate and reliable and gives more uniform results than the so-called permanent standards or semiquantitative methods, the extinction criterion has certain disadvantages which are inherent in the nature of its physiological optics and which prevent it achieving the accuracy of the best comparison photometry, because the human eye is unable to mark the disappearance of an object as critically as it can compare the brightness (color) of two specimens. For this reason the Electro-Scopometer more recently devised by Exton has still further increased the accuracy of scopometry.

METHODS FOR USING THE ANALYTICAL BALANCE

The balance should be kept level in a place of even temperature. In manipulating the balance all movements should be carefully made so that only a click is heard when the beam is raised and lowered. Nothing should be placed on or removed from the balance unless the mechanical supports are in place to take the weight off the knife edges. Exception may be made, however, in the case of weights smaller than 1 gram, if the pan rests are raised. With beam and pan rests having separate controls, as is the case in a good balance, the left hand manipulating the wheel moving the beam also controls the pan rests by bringing pressure of the side of the little finger against the push button. For use in keeping the balance parts free from dust and chemicals there should be provided a camel's hair brush about 1 inch wide. Objects which are to be accurately weighed should not be touched with fingers but handled with crucible tongs or otherwise. The zero point should be checked before each important weighing.

Determination of Equilibrium Point: Exact Method.—The exact limits to which the pointer moves on either side of the center of the scale are observed for an unequal number of swings and the mean of each set noted; then counting the excursions to the left "minus" and those to the right "plus," half the algebraic sum of the mean is taken as the equilibrium point. The amplitude of swing should be about 5 divisions on either side of the center mark. The first swing should be neglected. It is good practice to read three swings to the left and two to the right. If, for example, readings are as follows:

Divisions to Left		Divisions to Right
	− 5	+ 5.2
	− 4.6	+ 4.8
	− 4.3	
Sum	− 13.9	+ 10
Mean	− 4.63	+ 5

then the equilibrium point is $\dfrac{-4.63 + 5}{2}$ = +0.18 or 0.18 of a division to the right of the center.

Weighing.—Before weighings are started the true zero point should be determined by the above method. If the displacement of the zero point from the center of the scale is greater than one scale division, the balance should be readjusted. When weighing one of two procedures can be adopted. By adjustment of the weights and rider through trial and error the equilibrium point can be made to coincide with the true zero point. In the other less time-consuming method the equilibrium point is determined when it is brought to within several divisions of the zero point and calculation is made to the zero point by the use of the balance sensitivity values.

Sensitivity of Balance.—The sensitivity of a balance is defined as the number of scale divisions by which the zero point is displaced by an excess in weight of 1 milligram on one side or, otherwise stated, the weight required to cause 1 scale division displacement of the equilibrium point. With increasing loads the friction on the knife edges increases and the sensitivity diminishes, so that it is necessary to know the sensitivity for various loads, and determinations are made once for all and values kept on a card in the balance case for the following loads: no load, 1, 5, 10, 20, 50 grams. In practice the sensitivity for a given load is found by determining the variation made in the center of equilibrium when 1 milligram is added to the counterpoising weights, noting the number of divisions by which the center of equilibrium is changed, and then calculating the weight which would produce a variation of 1 scale division. A good analytical balance of 200 grams capacity has a sensitivity of 0.0003 gram or less without load and 0.0004 gram or less with full load. As readings are easily made to at least one-quarter of a scale division, it is seen that such a balance is well capable of weighing with an accuracy of 0.0001 gram and manufacturers commonly specify such balances as having a "sensitivity" of 0.1 milligram.

Weighing—Rapid Methods.—The procedures above outlined are too time-consuming for most ordinary work and there are several shorter methods by which almost equally accurate weighings can be made. It is important, however, with most of these that the analyst know his balance, particularly the sensitivity with various loads and the loss of amplitude taking place with each successive swing, and any method used should be occasionally checked by the longer procedure.

With a balance where one can regulate the initial throw of the pointer almost at will, an easy method consists in determining the zero point in terms of one set of opposite consecutive extreme excursion points (*e.g.,* four divisions to the left and three divisions to the right) and then with load in place adjusting weights so that the pointer again moves to the same two points, or points *near by* such that the difference between the opposite consecutive excursions is the same as the difference between the zero point excursion values (*e g,* with excursions at the zero point of four to left and three to right, the loaded balance could be assumed to be in equilibrium at the zero point when the pointer makes excursions of five to the left and four to the right, or 5.3 to left and 4 3 to right, etc.).

If the zero point of the unloaded balance has been adjusted to coincide with that of the scale, in the final adjustment of weights the loaded balance can also be brought to this ideal zero point by causing the excursion of the pointer to the right to be a certain fraction of a division less than the preceding excursion to the left, this value being previously determined as one-half the loss in amplitude occurring during one complete cycle over approximately the same range of swing.

In the method of equal swings the balance is adjusted so that the zero point is displaced to the right by half the amount of amplitude lost during one cycle. Then the weights are taken so that at the end of each swing to the right the pointer stops on the division corresponding to the starting point at the left. Any correction for known imperfect adjustment of zero point can be made by using the sensitivity values.

Weights.—Weights are always handled with the forceps. When the weighing is finished the empty places in the box and the rider position are noted and the weight recorded. This is confirmed by observation of the weights as they lie on the balance pan and may be reconfirmed by counting as weights are replaced in the box. Weights of good quality when new are accurate enough for ordinary work, but it may be desirable and for the most accurate work essential that the degree of error affecting the weights be known and be redetermined from time to time. Manufacturers of good weights allow a tolerance of ± 0.1 milligram in the case of 1-gram weights. In the calibration of weights, one of the 1-gram weights of the set, or better a standard 1-gram weight, is considered as the standard for the gram pieces and, at first, the 10-milligram weight as provisional standard for the milligram pieces. Combined use of standard and small calibrated pieces makes possible the callibration of the heavier pieces. By dividing the weight value obtained through balancing all the milligram weights together (they should theoretically weigh 1 gram) against the standard 1-gram weight by the arithmetical sum of the provisionally determined milligram values, a ratio is obtained which is used to correct the provisionally determined milligram values to the basis of the 1-gram standard. Because of the fact that the percentage error in the absolute weight of a 10-gram piece is likely to be less than in the case of a 1-gram piece in

the ordinary set of weights, it is probably advisable to reconvert all values on the basis of one of the 10-gram weights as standard.

Accessories.—In the so-called "chainomatic" balance final weight additions from 50 to 0.1 milligrams are made by simple adjustment in the length of a movable gold chain and this makes for more rapid and convenient weighing than is possible with the rider system. Weighing accessories consist of a spatula, a pair of matched watch glasses, glazed paper squares, a pair of crucible tongs, weighing bottles of several sizes, a large and a small camel's hair brush. When weighing is preceded by an ignition or hot drying operation, the object should be put in a desiccator while still warm, and after cooling there for twenty minutes or longer the weighing may be made. All objects must be at room temperature when weighed.

METHODS FOR THE PREPARATION OF STANDARD SOLUTIONS

All solutions, unless otherwise specified, are to be prepared of C.P or C P analyzed grades of chemical substances.

SULPHURIC ACID SOLUTIONS

Standard Normal Sulphuric Acid.—Principle.—The strength of a solution of sulphuric acid, slightly stronger than normal, is determined by titration against a known amount of sodium carbonate. It is then diluted to exactly normal and the dilution confirmed by titration.

Chemicals.—Sulphuric acid, C.P., specific gravity 1.84. Sodium carbonate, anhydrous, C P. special.

Reagents.—Methyl Orange.—Dissolve 0.1 gram of methyl orange in water and dilute to 100 c.c.

Sodium Carbonate Solution.—Dry the sodium carbonate in an oven at 105° C. for three to four hours. Weigh on an analytical balance 5.300 grams; dissolve in water; transfer quantitatively to a 100 c.c. volumetric flask and dilute to mark with water (normal solution).

Procedure.—1. In a 2 liter beaker containing about 1100 c c. water, slowly add about 33 c.c. sulphuric acid while stirring. Cool and fill a 50 c.c. buret with part of this solution.

2. Into a casserole pipet carefully 20 c.c. of the normal carbonate solution. Add 2 drops methyl orange reagent. Titrate with the acid from the buret until a faint pink color remains on stirring. Read the amount of acid required from the buret. Repeat the titration with another 20 c c. of the carbonate. They should check within 0 1 c.c.

3. Calculate the dilution as below. Then prepare exactly normal sulphuric acid and recheck this solution as above.

4. CALCULATION:

N = average of titrations in c c.
W = c c. of water to be added to the liter volumetric flask
$W = 50 (20 - N)$

5. Place W c c. of water in liter flask; add the sulphuric acid solution to the mark. Mix thoroughly. Place some of this solution in buret and titrate as before. If the solution is correct, *i e.*, exactly normal, 20 c.c. will neutralize the carbonate. If the average is less than 19.9 c.c., redilute as before and

confirm the titration. If the average is more than 20.1 c c., add several c c. of concentrated acid and repeat the preparation from the beginning

NOTE—Titrations between 19 9 and 20 1 may be accepted as sufficiently accurate for routine laboratory work.

Standard N/10 Sulphuric Acid.—With an accurately prepared normal solution, N/10, N/12, N/50, N/100, etc., solutions may be prepared by accurate dilution with distilled water. If the measurements are very carefully made, it is not usually necessary to make corrections for volume. If the solutions are not used very often, the normality should be checked occasionally by titration.

Eighteen Liters of N/12 Sulphuric Acid.—Many laboratories have a convenient 5 gallon container on hand to store a quantity of this acid. Also most laboratories have an accurate N/10 solution of sodium hydroxide. The following special method is given for those laboratories using large amounts of solution:

1. When first using the 5 gallon container, clean thoroughly, and with a large graduated cylinder measure rather carefully 18 liters of distilled water and make a permanent mark on the container at this volume. It may then be filled in subsequent times to this mark without measuring the quantity of water.

2. In a 50 c c. graduated cylinder, place exactly 43 c.c. of concentrated sulphuric acid. Pour this into the container, previously filled to the mark with distilled water, without contaminating the top, but, if so, rinse in with a little distilled water. Send a strong current of air through the solution for five minutes to mix. Take a 20 c.c. pipet and rinse with some of the solution.

3. Measure carefully 20 c c. of the solution into a 100 c.c. casserole. Add one drop only of methyl orange indicator solution. Titrate carefully with N/10 sodium hydroxide. Check the titration to within 0 5 c.c.

$$t = 20 - c\ c\ \text{of alkali used}$$
$$\text{Water to be added to each liter} = 60t.$$

4. Add this number of c.c. of distilled water to each liter of the solution or eighteen times this number to the whole container. Mix five minutes with air.

5. Titrate again, exactly as above. Check the titration. The titration should be 16.67 c.c. of N/10 sodium hydroxide.

6. In case a mistake has been made and the titration is less than 16.67 c c, a few drops of concentrated acid may be added and the process repeated as above.

7. If after the dilution the titration is still above 16.67, calculate the dilution again as above and again add the required amount of distilled water.

HYDROCHLORIC ACID SOLUTIONS

Standard Normal Hydrochloric Acid.—Principle.—This method depends upon the fact that when hydrochloric acid is boiled, a point is reached at which a constant boiling mixture is obtained, *i.e.*, the temperature of the mixture remains unchanged. At this point the distillate contains a definite amount of hydrochloric acid per c.c., depending upon atmospheric pressure. When such a hydrochloric acid solution is obtained, it becomes a simple matter to make from it any desired strength of acid by one exact weighing and dilution. Subsequent titrations are unnecessary.

Procedure.—In preparing constant boiling hydrochloric acid, approximately 1 liter of concentrated C.P. hydrochloric acid is diluted with water
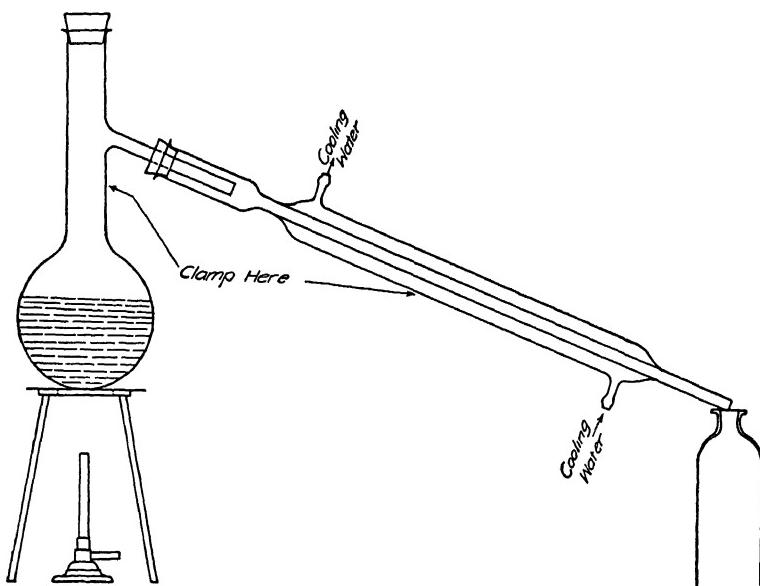
FIG. 281.—DISTILLING APPARATUS FOR CONSTANT BOILING HYDROCHLORIC ACID.

until the specific gravity is about 1.1 This acid is placed in a 2 liter side-arm round-bottomed distillation flask, stoppered with a sound cork stopper and the side arm connected, by means of a sound cork stopper, to a Liebig condenser (Fig. 281). The hydrochloric acid is then distilled, the flame being so regulated that about 3 to 4 c.c. per minute are collected in the receiving flask. After about two-thirds of the acid has been distilled, a clean, dry 1 liter bottle is substituted for the receiving flask, and the distillation continued until about 100 c.c. remain in the distilling flask. During the course of this part of the distillation, the barometric pressure is noted. This part of the distillate, about 400 c.c., constitutes the constant boiling fraction and should be preserved in

a cool place in an amber, ground-glass stoppered bottle. It will keep, if not opened too frequently, for several years.

The following table gives the amounts to be weighed for the preparation of 1 liter of normal hydrochloric acid:

Barometric Pressure in Millimeters	Weight in Grams of Constant Boiling Acid to Be Diluted to 1 Liter for a Normal Solution
770	.. 180.407
760	.. 180.193
750	179.979
740	179.766

Notes.—1. Weighings should be made in ground-glass stoppered weighing bottles of adequate capacity on an analytical balance.

2. Instead of weighing, acids may be measured with a pipet or measuring flask and standardized against some known alkali.

Standard N/10 and N/100 Hydrochloric Acids.—N/10 and N/100 hydrochloric acids may be prepared by accurately diluting the normal with distilled water.

N/10 may also be made by weighing one-tenth the amounts given in the table and diluting to 1 liter.

N/100 may also be made by weighing one-hundredth the amounts given in the table and diluting to 1 liter or dilute the N/10 standard 1:10 with distilled water. A new solution should be made up at least every two weeks.

A third method for the preparation of N/10 hydrochloric acid is as follows: Concentrated C. P. hydrochloric acid as marketed has a specific gravity which varies between 1.18 and 1.19 and the corresponding concentrations vary between 418 and 443 grams hydrochloric acid per liter. Make a solution slightly stronger than N/10 by using 9 c.c. concentrated acid per liter. For routine work it is convenient to make up 6 liters of solution.

To determine the exact strength of the solution, pipet 20 c.c. into a 100 c.c. Erlenmeyer flask, add 2 drops of methyl red indicator (0.02 per cent) and titrate with N/10 sodium carbonate prepared as on page 510 except that only one-tenth the amount of sodium carbonate is weighed out. The calculations for exact N/10 acid are the same as for sulphuric acid.

The solution keeps well, but if it is still in use at the end of a month's time, its strength should be checked.

SODIUM HYDROXIDE SOLUTIONS

Principle.—Since sodium hydroxide contains carbonate and is not suitable for many hydroxide solutions it is desirable to make a concentrated stock solution from which the carbonate will separate. This is diluted for use in the strength desired.

Procedure.—1. Weigh 1 kilogram of sodium hydroxide on a rough balance, and place it in a 2 liter rubber-stoppered flask. Care must be exercised because of the large amount of heat evolved. Add 1 liter distilled water and allow to dissolve with occasional shaking. Allow this to stand for a few days, when the carbonate will settle to the bottom, leaving a clear supernatant liquid. This solution will contain 70 to 75 grams sodium hydroxide for each 100 c.c. Dilution of this solution, considering it 73 grams per 100 c.c., will be sufficient for most sodium hydroxide solutions. For greater accuracy it may be titrated as follows:

2. Pipet accurately 5 c.c. of the stock sodium hydroxide into a 1 liter volumetric flask. Dilute to mark and mix. Pipet 20 c.c. of this diluted sodium hydroxide into a 150 c.c. Erlenmeyer flask, add 1 drop of phenolphthalein, heat to boiling, and titrate to the disappearance of the pink color with exact N/10 acid.

In case a normal solution of acid is available instead of the N/10 dilute 5 c.c. of the stock sodium hydroxide to 100 c.c. and proceed with the titration as above.

3. The calculation for either way is the same:

t = c.c. acid to titrate 20 c.c. of dilute alkali

Gm. sodium hydroxide per 100 c.c. stock sodium hydroxide = $4t$

4. To make any per cent sodium hydroxide solution desired:

Dilute $\dfrac{\text{per cent NaOH desired} \times 100}{\text{gram NaOH per 100 c c. stock solution}}$

= c.c. of the stock sodium hydroxide solution to 100 c c. with water.

For example:

10 per cent sodium hydroxide: $\dfrac{10 \times 100}{73}$ = 13.7 c.c. stock sodium hydroxide diluted to 100 c.c.

4.5 per cent sodium hydroxide: $\dfrac{4.5 \times 100}{73}$ = 6.2 c.c. stock sodium hydroxide diluted to 100 c.c.

N/10 Sodium Hydroxide Solution.—The sodium hydroxide should be as free as possible from carbonates; otherwise the solution will not have the same titrating value with all common indicators.

1. For each liter of N/10 sodium hydroxide desired, pipet off 7.5 c.c. of the clear concentrated solution described above and dilute to 1000 c.c. with distilled water. For routine work it is convenient to make up 6 liters of solution.

2. To determine the exact strength of the solution pipet 25 c.c. of the standard N/10 hydrochloric acid solution into a 100 c.c. Erlenmeyer flask, add 2 drops of 1 per cent alcoholic solution of phenolphthalein, heat to boiling, and titrate. Repeat the titration until the results check within 0.1 c.c.

3. To calculate the amount of distilled water which must be added to each liter of solution to make it exactly N/10, calculate as follows:

$$t = 25 \text{ c.c. of alkali used}$$

$$W = \text{amount of water to be added per liter}$$

$$W = 40\,t$$

4. Add this amount of water to a liter volumetric flask and fill to the mark with the hydroxide solution. Check by titration to make sure that the solution is exactly N/10.

5. Transfer to a clean, dry bottle and close with a rubber stopper. When set up for use the bottle should be protected from the carbon dioxide of the air by the use of a trap containing strong hydroxide solution.

6. If the solution is still in use at the end of a month's time its strength must be redetermined.

N/100 Sodium Hydroxide Solution.—Dilute the N/10 standard 1 : 10. Titrate against N/100 hydrochloric acid, using 0.1 per cent methyl red (containing a trace of methylene blue as a background color) as indicator. The N/100 hydrochloric acid is theoretically to be considered correct.

DECINORMAL POTASSIUM PERMANGANATE AND SODIUM OXALATE SOLUTIONS

Principle.—Decinormal sodium oxalate is prepared by accurately weighing pure sodium oxalate, dissolving and diluting to a definite volume. The potassium permanganate solution is prepared slightly stronger, titrated against the oxalate and finally diluted to exactly the normality desired.

Chemicals.—Potassium permanganate ($KMnO_4$). Sodium oxalate ($Na_2C_2O_4$) (Sorenson special). Sulphuric acid.

Procedure.—1. Dry out about 10 grams of the pure sodium oxalate in a drying oven at 105° C. for three to four hours. Weigh accurately 6.7 grams; dissolve in water and transfer quantitatively to a liter volumetric flask. Add 30 c.c. of concentrated sulphuric acid. Cool and dilute to mark. Mix. This is exactly N/10 sodium oxalate.

2. Dissolve about 3.5 grams potassium permanganate in about 1100 c.c. water. Let stand two weeks to permit oxidation of organic matter. Fill the buret with this solution.

3. Accurately pipet 20 c.c. of the N/10 sodium oxalate solution into a casserole and warm to about 75° C. Titrate with the potassium permanganate until a faint pink persists as the end-point. Note the amount of permanganate used. Repeat and check within 0.1 c.c.

4. CALCULATION:

t = average titration in c.c.

W = c c. water to be added to the volumetric flask

$W = 50 (20 - t)$

5. Place W c.c. water in a liter volumetric flask. Add the permanganate solution to the mark. Mix thoroughly. This should be exactly N/10 permanganate. It is well to repeat the titration as above when the titration should be 20.00 c.c.

NOTES.—The permanganate after titration should be kept in a dark place. The solution may change after it has been freshly prepared but after standing the strength is generally constant. If it is not exactly N/10 an appropriate factor may be used.

2. Permanganate solutions are so prone to change that it is sometimes more convenient to keep a strong solution on hand and dilute this to approximately the strength desired, followed by titration against a standard oxalate solution which keeps for a long time in a proper container; a factor is used in the titrations

Decinormal Oxalic Acid Solution.—1. Weigh on an analytical balance 3.1512 grams of special C.P. oxalic acid crystals for standardizing.

2. With a stream of water from a wash bottle, wash the crystals into a 400 c.c. beaker.

3. Dissolve in about 250 c.c. distilled water, gently heating.

4. Transfer to a 500 c.c. volumetric flask, carefully rinsing the beaker with successive amounts of water.

5. When cool, dilute to the mark and mix.

NOTE.—Oxalic acid crystals are neither efflorescent nor deliquescent and are to be transferred from the stock bottle directly to the watch glass. This solution does not keep well. The decomposition is accelerated by light.

STANDARD DECINORMAL SODIUM THIOSULPHATE SOLUTION

1. Since freshly dissolved sodium thiosulphate is decomposed by the carbonate always present in distilled water, use freshly distilled water boiled immediately before use.

2. Weigh, roughly, about 25 grams of C.P crystalline sodium thiosulphate for each liter of N/10 solution desired and dissolve in the required amount of water. After standing from eight to fourteen days standardize as follows:

About 1.5 grams of C.P. potassium iodide in 500 c.c. Erlenmeyer flask are dissolved in as little water as possible, 5 c.c. of hydrochloric acid (1 : 5)

added and then 20 c.c. (accurately measured) of the N/10 permanganate previously described. Iodine is liberated immediately and quantitatively and after diluting to about 250 c.c., is titrated at once by the addition of the thiosulphate solution from a buret. This solution is run in rapidly at first while the flask is vigorously shaken. After the yellow color begins to fade appreciably, add 1 c.c. of 0.5 per cent starch solution and continue the titration carefully to the complete disappearance of the blue color. If exactly N/10, this will require 20 c.c. of thiosulphate solution. It is not advisable to dilute to exact normality, but it is advisable to calculate the factor from the mean of three such titrations.

STANDARD SILVER NITRATE SOLUTION

1. Weigh 4.791 grams of C.P. silver nitrate on an analytical balance and dissolve in distilled water.

2. Transfer to a liter volumetric flask and make up to the mark with distilled water.

3. Mix thoroughly and keep in a brown bottle. 1 c.c. = 1 mg. chlorine.

DAKIN'S SOLUTION

Definition.—An aqueous solution of chlorine compounds of sodium, containing not less than 0.45 per cent and not more than 0 50 per cent of NaOCl, equivalent to from 0.43 to 0.48 per cent of available chlorine.

U. S. P. Method.—1. Chlorinated Lime,

Exsiccated Sodium Phosphate,

Water, each, a sufficient quantity,

To make.....................1000 c.c.

2. Assay the chlorinated lime as follows: Transfer to a mortar about 4 grams of chlorinated lime, accurately weighed in a tared weighing bottle, using 50 c.c. of distilled water. Triturate thoroughly, and pour the mixture into a graduated liter flask, rinsing the mortar with distilled water to make 1000 c.c. Stopper the flask, and allow it to stand for ten minutes. Shake thoroughly, add to 100 c.c. of the mixture 1 gram of potassium iodide and 5 c.c. of acetic acid, and titrate the liberated iodine with tenth-normal sodium thiosulphate, starch T.S. being used as indicator. Each c.c. of tenth-normal sodium thiosulphate corresponds to 0.003546 gram of available chlorine (Cl). Preserve in air-tight containers, in a cool, dry place.

3. Having determined its percentage of available chlorine, prepare the solution as directed below, using the quantities of chlorinated lime and exsiccated sodium phosphate indicated in the following table:

Per Cent of Available Chlorine in the Chlorinated Lime Used	Grams of Chlorinated Lime for Each 1000 c.c. of Solution	Grams of Exsiccated Sodium Phosphate for Each 1000 c.c. of Solution
20	29	38
21	28	36
22	27	34
23	26	32
24	25	30
25	24	28
26	23	26
27	22	24
28	21	22
29	20.5	21
30	20	20
31	19.5	20
32	19	20
33	18	20
34	17.5	20
35	17	20

4. Triturate the necessary amount of chlorinated lime, as indicated by the table, with 400 c.c. of water gradually added, until a uniform mixture results. Dissolve the required amount of exsiccated sodium phosphate, which has been recently dried, in 400 c.c. of water, heated to 50° C., and add this solution to the chlorinated lime mixture. Shake thoroughly and allow it to stand for fifteen minutes. Transfer the mixture to a filter, returning the first portions of filtrate until it runs through clear, and when no more liquid drains from the filter, wash the precipitate with sufficient water to make the product measure 900 c.c. Assay a portion as directed below, and dilute the remainder with sufficient water to make the finished solution contain 0.48 per cent of NaOCl.

Description and Physical Properties.—A colorless or faintly yellow liquid having a slight odor suggesting chlorine.

Tests for Purity.—Add about 0.02 gram of powdered phenolphthalein to 20 c.c. of Surgical Solution of Chlorinated Soda: no red color is produced on agitation (*maximum alkalinity*).

Add about 0.5 c.c. of phenolphthalein T.S. to 5 c.c. of the solution contained in a test tube; a momentary red flash is produced (*minimum alkalinity*).

Assay.—Dilute 25 c.c. of the solution, accurately weighed, with 50 c.c. of distilled water, add 1 gram of potassium iodide and 5 c.c. of acetic acid, and titrate the liberated iodine with tenth-normal sodium thiosulphate, using starch T.S. as indicator. Each c.c. of tenth-normal sodium thiosulphate corresponds to 0.003723 gram of NaOCl.

Preserve in well stoppered bottles, preferably for not more than seven days, in a cool place, protected from light.

CHAPTER XXXI

METHODS FOR THE CHEMICAL EXAMINATION OF THE BLOOD

PREPARATION OF GLASSWARE

1. Glassware should be washed as soon after use as possible. Wash with tap water, using a solution of soap made by dissolving a soap powder or chips in tap water. Rinse thoroughly with tap water and finally with distilled water and allow to dry.

2. A tall crock or cylinder full of tap water with a layer of cotton in the bottom should be kept near to receive soiled pipets immediately after using. To wash pipets hold them in the flowing tap water, or better, use a water suction pump attached to the faucet. Place the one end of the pipet in the rubber tube connected with the pump and the other end in a container of tap water and allow the pump to suck the water from the container through the pipet for about half a minute. Remove the pipet from the rubber tube and allow a little distilled water to run through the pipet, then put aside to dry.

3. When glass retains a cloudiness that cannot be removed by means of washing with soap, use potassium dichromate sulphuric acid cleaning solution, made as follows:

Commercial potassium dichromate.......... 5.0 gm.
Commercial sulphuric acid................ 500 c.c.

Add sufficient water to dissolve the dichromate with heat before adding the acid.

Fill beakers and flasks with cleaning solution and place pipets in a tall cylinder full of solution and allow to remain twenty-four hours.

4. The cleaning fluid can be used repeatedly and when it appears to lose its strength add more potassium dichromate and sulphuric acid.

COLLECTION OF BLOOD

1. Blood is usually taken from a vein at the elbow with a sterile syringe, the technic being described and illustrated in Chapter XXIV. Blood may also be taken from a finger, if a micromethod of analysis is to be conducted as in blood sugar determinations. Umbilical cord blood may be secured at birth or specimens obtained from infants and young children by puncture of the external jugular veins or superior longitudinal sinus, the methods being described and illustrated in Chapter XXIV.

2. After standardization of diet and exercise, a pneumatic tourniquet is applied to the arm at a pressure sufficient to obstruct the venous but not the arterial circulation. Blood is withdrawn with a syringe and needle and transferred to an oxalated tube.

3. Specimens, except those for determination of calcium, icterus index, bilirubin and bromsulphthalein, are to have an anticoagulant added

4. Sodium oxalate is the most generally useful anticoagulant; an excess should be avoided.

5. Blood sugar is rapidly destroyed (glycolysis) on standing and specimens intended for sugar determination or sugar and other determinations should have sodium fluoride (C.P. powder) added as a preservative and anticoagulant in the proportion of 60 milligrams per 10 c.c. blood if the determination cannot be made almost immediately. As sodium fluoride is not very soluble, it is necessary to mix thoroughly to prevent clotting. If the blood is on the point of coagulating, it is well to add oxalate also. *Fluoride should not be added to specimens intended for urea determination as results will be too low.* The addition of thymol to the fluoride is recommended for preventing or greatly inhibiting glycolysis for several days. Chlorbenzol may be used as a preservative for specimens to be kept up to ten days or longer for sugar determinations

6. Twelve c.c. of whole blood will just suffice for a determination of non-protein nitrogen, urea nitrogen, uric acid, creatinine, sugar and chlorides.

7. **Preparation of Oxalated Tubes.**—Pipet 0.5 c c. of a hot saturated solution of sodium oxalate solution into test tubes of 25 c.c. capacity, fitted with rubber stoppers. At low heat and with constant rotation evaporate the solution, leaving the dry sodium oxalate deposited in a finely divided state about the sides of the tubes.

8 **Procedure for Venipuncture.**—Before or three hours after breakfast, place the subject in dorsal recumbent posture and apply the tourniquet above the belly of the biceps. Sponge the cubital fossa with alcohol and allow to dry. Adjust the pressure of the tourniquet so that the radial pulse can just be felt. Locate the vein (median cephalic or median basilic), the more prominent to palpation, and fix it between the thumb and finger. Insert the needle into the vein with one thrust, with bevel down and nearly parallel to the skin surface. Turn bevel up. Collect 5 to 10 c.c. of blood in the syringe. Release the tourniquet and withdraw the needle. Transfer blood immediately to oxalated tube. Insert the rubber stopper into tube and invert several times. Place alcohol sponge over puncture point and press for one or two minutes.

NOTES.—1. When possible, blood samples are examined immediately after withdrawal. However, blood may be preserved in paraffin-coated tubes at a temperature of 0° C. to 5° C. for several days with practically no change in the usually sought constituents except carbon dioxide and hydrogen ion concentration.

2. A blood sample for the determination of hydrogen ion concentration is

drawn in a special manner, in a centrifuge tube or heavy-walled test tube, under paraffin oil to avoid contact with air, and without stasis in the vein (Fig. 282).

3. When serum is required for the determination, as for calcium and Van den Bergh, the oxalate is omitted and the blood allowed to clot.

4. *In the following pages whenever directed to "dilute to the mark," distilled water is to be used unless some other solution is especially mentioned. The word "water" always implies distilled water.*

5. The chemicals used are all of the grade of C.P. Analyzed Chemicals, unless otherwise mentioned.

6. The table on pages 522–523 indicates the amounts of blood or plasma ordinarily required for the various determinations according to the methods indicated; it also shows the normal values in *milligrams per 100 c c.* unless otherwise stated.

PREPARATION OF PROTEIN-FREE FILTRATE

Principle.—The proteins of blood are completely removed by filtration following precipitation with tungstic acid, which is formed by the interaction of sodium tungstate and sulphuric acid.

Reagents.—1. Sulphuric acid (N/12): Prepared as described in Chapter XXX.

2. Sodium tungstate: Not all brands, even though labeled C.P., are suitable. Some may contain too much sodium carbonate. This may be tested as follows: To 10 c.c. of 10 per cent sodium tungstate add 1 drop of phenolphthalein and titrate with N/10 hydrochloric acid to the first point of red fading. Each c c. of hydrochloric acid is equivalent to 1 06 per cent sodium carbonate. The amount of acid used should not exceed 0.4 c.c. if the specimen is to be satisfactory for use.

Paraffin Oil
Potassium Oxalate

FIG 282 —TUBE USED IN COLLECTING BLOOD FOR DETERMINATION OF PH OR CARBON DIOXIDE.

(Van Slyke and Cullen, *J. Biol Chem,* 1917, 30·289)

The sodium tungstate must be very easily soluble in water. If it is not easily soluble, its solutions are not alkaline to phenolphthalein and this shows that the product contains paratungstates. Baker's C P. analyzed special sodium tungstate is satisfactory.

3. The filter paper should be ammonia-free; diameters 11 to 12.5 centimeters. Whatman No. 2 is recommended.

Procedure.—Haden's Method.—1. Transfer 1 volume of oxalated whole blood to a flask having a capacity fifteen to twenty times that of the volume taken. In ordinary practice 2 or 4 c.c. of blood, depending on the number

Determination	Method	Normal Value	For One Determination	Blood Desired *
Sugar †	Folin and Wu Folin-Micro	80 to 110	2 c.c. W.B. 0.1 c.c. W.B.	5 c.c. 0.1 c.c.
Nonprotein nitrogen..........	Folin and Wu	25 to 40	2 c.c. W.B.	5 c.c.
Urea nitrogen ‡..........	Van Slyke and Cullen	10 to 15	3 c.c. W.B.	7 c.c.
Uric acid..........	Brown	2 to 4	2 c.c. W.B.	5 c.c.
Creatinine	Folin and Wu	1 to 2	4 c.c. W.B.	5 c.c.
Carbon dioxide capacity §..........	Van Slyke	Adults, 53 to 77 vol. per cent Infants, 43 to 65 vol. per cent	1 c.c. P.	5 c.c.
Amino acid nitrogen	Folin	6 to 8	2 c.c. W.B.	5 c.c.
Fibrinogen..........	Wu	0.2 to 8.5 per cent	1 c.c. P.	6 c.c.
Albumin.......... Globulin.......... A : B ratio	Wu	4.5±0.5 per cent 2.0±0.4 per cent 1.5 to 3.0 : 1	2 c c. P.	10 c.c.
Cholesterol..........	Leiboff	140 to 180	0.25 c.c. W.B.	5 c.c.
Total fat..........	Bloor	600 to 700	2 c.c. W.B.	5 c.c.
Creatine	Folin and Wu	3 to 7	4 c.c. W.B.	5 c.c.
Total acetone bodies..........	Van Slyke and Fitz	1 to 3	10 c.c. W.B.	11 c.c.
Chlorides..........	Whitehorn	450 to 520	4 c.c. W.B.	5 c.c.
Calcium ‖	Clark and Collip	9 to 11	2 c.c. S.	13 c.c.

Inorganic phosphorus ¶	Benedict and Theis	Adults, 2.5 to 4.5 Infants, 4 to 6	2 c.c. P.	8 c.c.
Bilirubin	Van den Bergh-McNee-Hall	0.1 to 0.3	3 c.c. S.	8 c.c.
Icterus index	Meulengracht and Bernheim	4 to 6	2 c.c. S.	5 c.c.
Bromsulphthalein	Rosenthal and White	20 to 50 per cent in 5 minutes	4 c.c. S. each	8 c.c. each
PH **	Cullen and Myers	7.3 to 7.5	0.1 c.c. P.	5 c.c.
Oxygen capacity ††	Van Slyke and Stadie	Men, 21 vol. per cent Women, 18 vol. per cent	2 c.c. W.B.	5 c.c.
Venous oxygen unsaturation §	Van Slyke and Stadie	2.5 to 9.0 vol. per cent	4 c.c. W.B.	12 c.c.
Methemoglobin ††	Stadie (Spectroscopic)	0 quantitative 0 qualitative	4 c.c. W.B. 2 c.c. W.B.	10 c.c. 5 c.c.
Carbon monoxide ††	Sayers and Yant	0	1 c.c. W.B.	5 c.c.

Normal values above refer to venous blood. Unless otherwise specified, values are expressed in milligrams per 100 c.c. W.B. = whole blood. P. = plasma. S. = serum.

* Provides excess amounts for recheck and duplicate determinations.

† The micromethod may be found useful when ven puncture is difficult. As capillary blood is used, the normal values with this method may be 5 or 10 milligrams higher than normal values for venous blood.

‡ In cases where it is impossible or impracticable to obtain a specimen of blood, urea may be determined in saliva. The patient's mouth should be clean and should be rinsed out with half a glass of water before collection is started. Send to the laboratory (about 20 c.c.) at once.

§ Collect without stasis under neutral paraffin oil in a special tube containing the necessary amount of oxalate. Send to laboratory at once.

‖ As tap water contains considerable calcium, the bloods are to be collected in syringes which have been sterilized in distilled water. Do not put in ice box.

¶ Send to laboratory at once. On standing organic phosphates break down to form inorganic phosphates and results are too high.

** To be collected by the laboratory. About four hours' work is required to prepare the necessary solutions. This is a colorimetric method and is not recommended as giving true values.

†† Send to the laboratory at once.

of determinations to be performed, are transferred to a 100 c.c. Erlenmeyer flask. Ostwald-Folin pipets (Fig. 283) should be used. They should be drained against the sides of the test tubes ten seconds and then blown clean.

2. Add 8 volumes of N/12 sulphuric acid, the contents of the flask being shaken during the addition. In practice, 16 or 32 c.c. are added from an automatic pipet. The blood is laked very rapidly and becomes dark brown through the conversion of hemoglobin into acid hematin. Allow to stand at least one minute.

3. After the laking is complete, add 1 volume of the 10 per cent solution of sodium tungstate. In practice 2 or 4 c.c. are added from an automatic pipet. Close the mouth of the flask with a rubber stopper and shake thoroughly. The coagulum should be dark brown, with little or no frothing.
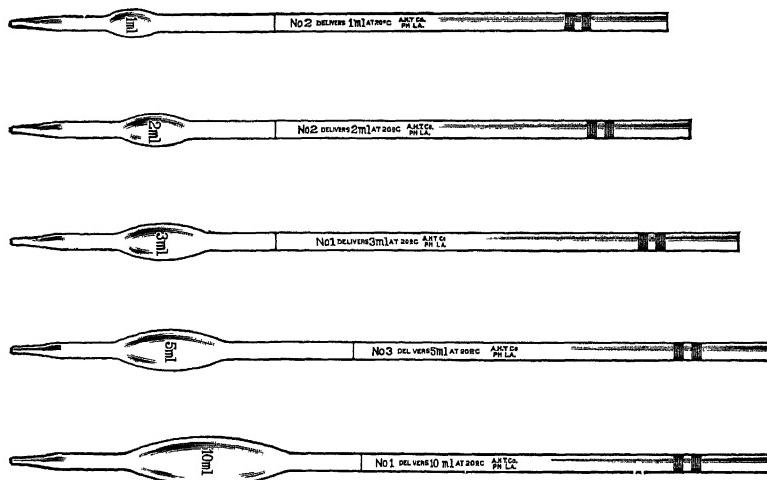
FIG. 283 —OSTWALD-FOLIN PIPETS.

2. Pour the mixture on a filter large enough to hold the entire contents. The filtration should be begun by pouring the first c.c. down the triple portion of the filter and withholding the remainder until the whole filter is wet. Collect the filtrate in a clean, dry test tube. The filtrate should be perfectly clear. It may be necessary to refilter the first 2 or 3 c.c. by returning it to the funnel, although this is seldom required. Instead of filtration the mixture may be centrifuged. For the filtration of larger amounts, the Gooch crucible (Fig. 284) may be employed.

5. If the filtrate is not to be used within a short time it should be placed in the ice box. If it is to be kept longer than two days, a few drops of toluene should be added to prevent bacterial decomposition. Filtrates from oxalated blood may be kept overnight in the ice box without appreciable loss of sugar or uric acid; standing several days has little effect on the nonprotein nitrogen, creatinine or creatine values.

NOTES.—1. If there is much foaming and the coagulum assumes a brownish-pink instead of a dark brown color, it is usually because too much oxalate is present. In such a case the sample can generally be saved by adding 10 per cent sulphuric acid, one drop at a time, shaking vigorously after each drop, and continuing until there is practically no foaming and until the dark brown color has set in.

2. If the filtrate is not clear, the precipitate and the filtrate should be returned to the flask and 10 per cent sulphuric acid added as above to complete the protein precipitation.

3. The filtrate should be nearly neutral when the reagents are properly adjusted. With Congo red the filtrate should give a negative test and with blue litmus a positive test. Excess acidity will result in precipitation of uric acid and will give trouble in the determination of sugar by the new Folin method. To be suitable for the determination of sugar by the Folin method, 10 c.c. of filtrate on titration with $N/10$ sodium hydroxide and 1 drop of phenolphthalein should give an end-point with about 0.2 c.c. alkali. Haden reports 0.42 c.c. $N/10$ sodium hydroxide as an average titration figure for 10 c.c. filtrate.

Folin-Wu Method.—1. One volume of whole blood.

2. Dilute with 7 volumes of distilled water (Folin-Wu blood pipet, Fig. 90). Mix to lake.

3. Add 1 volume of 10 per cent sodium tungstate solution and mix.

4. Add slowly (drop by drop) with constant shaking, 1 volume of two-thirds normal sulphuric acid solution prepared as described in Chapter XXX.

FIG 284—FILTRATION WITH THE GOOCH CRUCIBLE

5. To obtain the maximum recovery of uric acid, this step should be modified as follows: Add slowly (drop by drop) with constant shaking four-fifths of the required two-thirds normal sulphuric acid and let the mixture stand for twenty to thirty minutes before adding the remaining one-fifth of sulphuric acid. About 3 per cent more uric acid is obtained.

6. Proceed as with the Haden modification of the Folin-Wu method.

7. This method is to be used for recovery experiments in checking different methods.

8. Glycolysis does not take place in laked blood, so when time is lacking for the preparation of a protein-free filtrate, the procedure can be safely interrupted after the addition of the 7 volumes of water.

The sulphuric acid should be added with constant shaking, otherwise localized zones of hyperacidity will result in precipitation of uric acid.

DETERMINATION OF UREA NITROGEN

Principle.—Protein-free blood filtrate is incubated with urease and phosphate buffer solution. The resultant solution is nesslerized and compared colorimetrically with a similarly treated standard urea solution.

Reagents.—1. UREASE.—Place 15 grams of jack bean meal and about 5 grams of permutit in a large Erlenmeyer flask and add a mixture of 16 c.c. of alcohol and 84 c.c. of water. Shake gently but continuously for ten to fifteen minutes. Allow to stand overnight and filter into small flasks. Keep in refrigerator, tightly stoppered.

2. PHOSPHATE BUFFER.—Dissolve 14 grams of sodium pyrophosphate and 2 grams of the metaphosphoric acid in water and dilute to 250 c.c.

3. UREA NITROGEN STOCK SOLUTION.—Dissolve 0.1286 gram of urea in water and dilute to 200 c.c. (5 c.c. contains 1.5 milligrams urea nitrogen).

4. UREA NITROGEN STANDARD FOR USE.—Place 5 c.c. of the stock urea solution in a 100 c.c. volumetric flask and dilute to mark (5 c.c. contains 0.075 milligram urea nitrogen).

5. NESSLER SOLUTION.—See directions under Determination of Nonprotein Nitrogen, page 530.

Procedure (Karr).—Into an ordinary test tube marked S, pipet 5 c.c. of the standard solution of urea; into another tube marked B, pipet 5 c.c. of the protein-free blood filtrate. Into each add 5 drops of the urease solution and 5 drops of the phosphate buffer solution. Place tubes in water bath at 50° C. for ten minutes. At the end of this time transfer quantitatively the solutions in each tube to the graduated tubes (marked S and B). Wash out the tube twice with about 5 c.c. of water into the graduated tube. Add 1 drop of 1 per cent gum ghatti solution and dilute to the lower mark with water. From the reservoir bottle add the Nessler solution of the 25 c.c. mark. Mix by inverting. Compare in colorimeter.

CALCULATION:

$$x = \text{milligrams urea nitrogen in 100 c.c. of blood}$$

$$s = \text{reading of the standard (contents of tube } S)$$

$$R = \text{reading of the blood filtrate (contents of tube } B)$$

$$x = \frac{15s}{R} = \frac{300}{R} \text{ if standard is set at 20}$$

or set the cup of the unknown at 15 millimeters when the reading of the standard cup equals milligrams of urea nitrogen per 100 c.c. of blood.

NOTES.—1. The normal range is about 10 to 15 milligrams urea nitrogen in 100 c.c. of blood.

2. The tubes in which the filtrate and standard are incubated must be kept clean and never used to contain the Nessler solution.

3. With bloods known or thought to have a high urea nitrogen content, use less filtrate and make corresponding calculation. For a large number of determinations at one time, an artificial standard may be used. The amount of urea nitrogen in the blood is affected by diet. Urea nitrogen is increased above normal in kidney insufficiency when the kidney is secondarily affected. Retention may be said to exist when the concentration reaches 20 milligrams in 100 c.c. of blood.

MICROMETHOD FOR DETERMINING UREA NITROGEN

Principle.—Protein-free blood filtrate is incubated with urease and a buffer solution. The resultant solution is nesslerized and compared colorimetrically with a similarly treated standard urea solution.

Reagents.—1. UREASE.—To 16 c.c. of alcohol add 84 c.c. of water. To this add 15 grams of jack bean meal and 5 grams of permutit. Shake this mixture for fifteen minutes, and allow to stand over night in a refrigerator. Filter and keep in refrigerator.

2. PHOSPHATE BUFFER.—Dissolve 14 grams of sodium pyrophosphate and 2 grams of metaphosphoric acid in water and dilute to 250 c.c.

3. UREA NITROGEN STOCK.—Dissolve 0.1286 gram of urea in water and dilute to 200 c.c. (5 c.c. contains 1.5 milligrams urea nitrogen).

4. UREA NITROGEN STANDARD.—Dilute 5 c.c. of urea stock solution to 100 c.c. with water. (1 c.c. contains 0.015 milligram urea nitrogen.)

5. NESSLER SOLUTION.

6. TUNGSTIC ACID SOLUTION.—To 16 c.c. of N/12 sulphuric acid add 80 c.c. of water; then 2 c.c. of 10 per cent sodium tungstate solution.

Procedure (Keller).—1. Place 0.8 c.c. of tungstic acid solution in a 15-c.c. centrifuge tube. Prick finger with a lancet so that the blood flows freely. Using a 0.2-c.c. serological pipet, collect 0.2 c.c. of blood. Introduce into the centrifuge tube and rinse pipet. Stopper and mix. Centrifuge at high speed for five minutes.

2. Into a small test tube "B" pipet 4 c.c. of the supernatant fluid. Into another small test tube "S" pipet 1 c.c. of urea nitrogen standard solution. To each tube add 3 drops of urease and 3 drops of buffer solution.

3. Digest for ten minutes at 50° C.

4. Transfer contents to respective graduated tubes, rinsing with water and diluting to 9 c.c. mark. Add Nessler's solution to 10 c.c. mark.

5. Compare colorimetrically.

6. CALCULATION —In a Duboscq type colorimeter, set the unknown sample at 15 millimeters, when 1.25 times the reading of the standard gives milligram of urea nitrogen per 100 c.c. of blood.

DETERMINATION OF UREA NITROGEN

Principle.—Urea in whole blood is hydrolized by urease and the ammonia is aerated into a standard acid solution and estimated by titration.

Reagents.—*Urease.*—Urease 0.1 gram tablets (Squibb). Squibb's urease is made from jack beans and is compounded with molecular proportions of phosphates of potassium as recommended by Van Slyke and Cullen.

Hydrochloric Acid N/100.—Should be freshly prepared or restandardized every three weeks.

Sodium Hydroxide N/100.—Should be freshly prepared or restandardized every three weeks. More frequently it should be checked against the N/100 hydrochloric acid.

Potassium Carbonate, Saturated Solution.—Dissolve U.S.P. potassium carbonate in distilled water, 90 grams anhydrous powder or 108 grams crystals ($K_2CO_2 \cdot 1\frac{1}{2}H_2O$) to form 100 c.c. solution. A blank test for ammonia should be negative.

Methyl Red Indicator, 0.02 per cent.—Dissolve 0.1 gram methyl red in 250 c.c. of 95 per cent ethyl alcohol and then add 250 c.c. of distilled water.

Caprylic Alcohol, pure.

Procedure (Van Slyke and Cullen).—1. Pipet 3 c.c. of oxalated whole blood into a 75 c.c. lipless Pyrex test tube (200 × 25 mm), A.

2. Add one powdered 0.1 gram urease tablet (Squibb).

3. Stopper and let stand at room temperature for thirty minutes, or at a temperature of 40° to 55° C. for fifteen minutes.

4. Then with apparatus set up for aeration, quickly add 2 or 3 drops of caprylic alcohol, and

5. Add approximately 5 c.c. of saturated potassium carbonate solution.

6. Drive off the ammonia by aspiration into another large test tube, B, containing 15 c.c. of N/100 hydrochloric acid and 4 drops of methyl red indicator. During the first two minutes the air current should be slow, and thereafter as rapid as the apparatus will stand. Aerate for thirty minutes to one hour depending on the air current used.

7. Titrate the excess acid with N/100 sodium hydroxide, using the aeration tube as stirrer.

8. CALCULATION: Each c.c. of acid neutralized by ammonia represents 4.67 milligrams urea nitrogen or 10 milligrams urea per 100 c.c. blood.

(15 — c.c. N/100 NaOH used) × 4.67 = milligrams urea N per 100 c.c. blood.

The table from Haden simplifies calculations.

Notes.—The lipped test tubes used for the nonprotein nitrogen determination should not be used, because Nessler's reagent leaves on the glass a film of mercury compound which inactivates urease. If these tubes must be

TABLE FOR CALCULATION OF BLOOD UREA N

3 c.c. blood and 15 c.c. N/100 HCl being used, the table shows blood urea N in milligrams per 100 c.c. corresponding to the number of c.c. of N/100 NaOH required to titrate the excess N/100 HCl.

NaOH Required, c.c.	0.0	0.1	0.2	0.3	0 4	0.5	0.6	0.7	0.8	0.9
0	70.05	69.58	69.12.	68.65	68.18	67.71	67.25	66.78	66.31	65.85
1	65.38	64.91	64.45	63.98	63.51	63.05	62.58	62.11	61.64	61.18
2	60.71	60.24	59.78	59.31	58.84	58.38	57.91	57.44	56.97	56.51
3	56.04	55.57	55.11	54.64	54.17	53.71	53.24	52.77	52.30	51.83
4	51.37	50.90	50.44	49.97	49.50	49.04	48.57	48.10	47.63	47.17
5	46.70	46.23	45.77	45.28	44.83	44.36	43.90	43.43	42.96	42.50
6	42.03	41.56	41.10	40.63	40.16	39.70	39.23	38.76	38.29	37.83
7	37.36	36.89	36.43	35.96	35.49	35.03	34.56	34.09	33.62	33.16
8	32.69	32.22	31.76	31.25	30.82	30.36	29.89	29.42	28.95	28.49
9	28.02	27.55	27.09	26.62	26.15	25.68	25.22	24.75	24.28	23.82
10	23.35	22.88	22.42	21.95	21.48	21.01	20.55	20.08	19.61	19.15
11	18.60	18.21	17.75	17.28	16.81	16.35	15.88	15.41	14.94	14.48
12	14.01	13.54	13.08	12.61	12.14	11.68	11.21	10.74	10.27	9.81
13	9.34	8.87	8.41	7.94	7.47	7.00	6.54	6.07	5.60	5.14
14	4.67	4.20	3.74	3.27	2.80	2.34	1.87	1.40	0.93	0.47

used, they should be washed with nitric acid and then thoroughly rinsed with water.

The tubes may be conveniently held for aeration in a holder made by boring holes about 3 inches deep and 1⅜ inches wide in a heavy block of wood. The block may be made to hold eight pairs of tubes. An odd hole at one end serves to hold a tube with acid for washing the air. The bubbling ends of the aeration tubes are perforated by a number of pin holes to assure maximum distribution of air and should be inserted to extend nearly to the bottoms of the test tubes. The rubber tubing used for the connections needs to be rinsed with water before being used the first time, in order to free it from talc which may contain ammonia. Tubing should also be washed, if the apparatus has stood a long time without being used.

If a test tube and stopper are used as container, first for the strong carbonate solution, then during a subsequent aeration, for the N/100 standard acid solution, the tube or stopper, no matter how thoroughly washed with distilled water, is likely to carry a trace of alkali into the highly dilute acid and appreciably affect the result. If a tube that has been used for an alkaline solution is to be employed for standard acid, both tube and stopper are washed first with dilute acid, then with water.

Caprylic alcohol and potassium carbonate should be added quickly and the tubes corked at once to prevent loss of ammonia.

The solution from which the ammonia is driven must contain at least 1 gram of potassium carbonate for each 2 c.c. of solution, and if so, the volume of the solution can be increased up to 25 c.c. without appreciably altering the time required to drive off the ammonia.

The air used for aeration should be bubbled through dilute sulphuric acid to remove atmospheric ammonia. Suction is preferable to pressure, but pressure may be used, if precautions are taken to see that the corks are tight.

The column of acid in the tube should be at least 50 millimeters high in order to insure complete absorption of ammonia.

Eight determinations may be run at one time, in which case a slower current and one hour aeration will probably be desirable. At the end of the aeration period do not suddenly turn off the air current, or solutions will be sucked over into adjacent tubes; disconnect the separate tubes beginning at the end farthest from the air line.

A blank determination should be run with each new lot of reagents and the necessary deduction made, if ammonia is found. Fresh blood contains so little free ammonia that it may be disregarded.

If a blood contains more than 70 milligrams urea N per 100 c.c. and all the acid is neutralized, the determination must be repeated with a smaller sample of blood, and as the adjacent determination in the rack is spoiled, it also must be repeated. If one observes change in color of the indicator from red to pink, the determination can be saved by stopping aeration and adding more standard acid.

Sodium fluoride should not be added to bloods intended for accurate urea determination by the urease method, as fluoride has an inactivating effect on urease. Because of ammonia formation it is desirable that analyses be performed within several hours after blood is drawn. Bloods that have stood one day at room temperature show values which are too high by several milligrams urea N per 100 c.c. Standing over night in an ice box is permissible in routine work. Some significance may be attached to urea determinations in bloods obtained from refrigerated bodies within twenty-four hours postmortem; under such conditions, however, the creatinine determination is more accurate.

DETERMINATION OF NONPROTEIN NITROGEN

Principle.—The protein-free blood filtrate is treated with an acid mixture, which converts the nitrogen into ammonia. The solution is nesslerized and read against a standard ammonium sulphate similarly treated.

Reagents.—1. ACID DIGESTION MIXTURE.—Mix 300 c.c. of C.P. syrupy phosphoric acid (85 per cent) with 100 c.c. concentrated C.P. sulphuric acid. Transfer to a tall cylinder, cover well to exclude absorption of ammonia, and

set aside for sedimentation of calcium sulphate. At the end of a week or so, pipet off 50 to 100 c.c. of the supernatant fluid. To 100 c.c. of the clear acid add 10 c.c. of 6 per cent copper sulphate and 100 c.c. distilled water. Ten c.c. of a 1:10 dilution should be neutralized by 9 to 9.3 c.c. of Nessler's reagent, phenolphthalein being used as indicator.

2. NESSLER'S REAGENT (Folin's modification).—This reagent is essentially a solution of the double iodide of mercury and potassium containing sodium hydroxide.

3. MERCURIC POTASSIUM IODIDE SOLUTION.—Transfer 150 grams potassium iodide and 110 grams iodine to a 500 c.c. Florence flask. Add 100 c.c. distilled water and an excess of metallic mercury, 140 to 150 grams.

Shake the flask continuously and vigorously until the dissolved iodine has nearly all disappeared. The solution becomes hot. When the red iodine solution has become visibly pale, though still red, cool in running water, and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide.

Separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a final volume of 2 liters. If the cooling was begun in time the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water.

4. SODIUM HYDROXIDE SOLUTION.—From a completely saturated sodium hydroxide solution, containing about 55 grams of sodium hydroxide per 100 c.c., decant the clear supernatant liquid. To 1 volume of sodium hydroxide add 4.5 volumes of distilled water. Determine by titration that a 10 per cent solution has been obtained with an error of not over 5 per cent.

WORKING SOLUTION.—Transfer to a large bottle 350 c.c. of 10 per cent sodium hydroxide. Add 75 c.c. of the potassium mercuric iodide solution. Add 75 c.c. of distilled water. Titrate, using phenolphthalein as an indicator. If the solution is substantially correct, 20 c.c. N/10 hydrochloric acid will be neutralized by 11 to 11.5 c.c. of Nessler's solution which has been diluted 1:10. If an end-point is obtained below 11 c.c., the Nessler solution is too alkaline and turbidity is likely to occur.

STANDARD AMMONIUM SULPHATE SOLUTION.—Ammonium sulphate (C.P., special, pyridine free) should be dried in hot air for one half hour at 110° C and then allowed to cool twenty minutes in a desiccator. Weigh on an analytical balance 0.4716 gram. Wash into a beaker to dissolve, then wash into a liter volumetric flask. Add 1 c.c. concentrated C.P. hydrochloric acid (to prevent growth of molds). Dilute to the mark with distilled water. Keep in a well-stoppered bottle labeled 3 c.c.=0.3 milligram of nitrogen.

NOTES ON NESSLERIZATION.—1. A slightly excessive concentration of alkali at once precipitates the colloidal colored ammonium compound. Local zones of excessive alkalinity occurring coagulate part of the solution. The Folin

reagent is dilute enough so that localized zones of alkalinity ordinarily do not occur.

2. Turbidity in the final solution, aside from that due to silicon dioxide, is ordinarily due to the fact that the Nessler's solution is too strongly alkaline or the acid digestion mixture is too weak or too much sulphuric acid has been lost during digestion.

3. Large amounts of sulphates lead to the precipitation of the colloidal colored ammonium compound. It is for this reason that phosphoric acid is used in the digestion mixture. The most disturbing impurity is magnesium in any form and it is because of its magnesium content that tap water cannot be used for the preparation of solutions which are to be nesslerized.

4. The use of funnels is primarily to prevent loss of sulphuric acid so that the alkalinity of the nesslerized standard and unknown shall be the same. The greater the alkalinity the deeper is the color. In eliminating the loss of sulphuric acid fumes there is also eliminated all danger of losing ammonia together with those fumes.

Procedure (Folin and Wu).—1. Pipet 5 c.c. of protein-free blood filtrate into a dry, lipped, thin-walled, 75 c.c. Pyrex test tube (200 by 25 millimeters) graduated at 35 c.c. and 50 c.c.

2. Add 1 c.c. of the sulphuric-phosphoric acid digestion mixture and to prevent bumping add a *dry* Pyrex glass bead or a quartz pebble (may be omitted).

3. Clamp the test tube in a test tube support and boil vigorously with the micro-burner until the characteristic dense acid fumes fill the tube. This will occur in from three to seven minutes, depending on the size of the flame.

4. Turn down the flame so that the contents are just visibly boiling and close the mouth of the tube with a short funnel. Continue the heating for two minutes from the time the fumes begin to be unmistakable, even if the solution has become clear and colorless at the end of twenty to forty seconds, as it usually does. If the oxidations are not visibly finished at the end of two minutes, the heating must be continued until the solution is nearly colorless.

5. Turn out the flame, remove the funnel, and allow to cool.

6. When cooled to room temperature add distilled water to the 35 c.c. mark.

7. Add Nessler's reagent to the 50 c.c. mark, insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion, giving a crystal clear fluid above a white sediment (silica). If the sediment is colored, the nesslerization was not successful and the determination must be discarded. The unknown and the standard should be nesslerized at approximately the same time.

8. Prepare the standard as follows: Pipet 3 c.c. of ammonium sulphate standard into a 100 c.c. volumetric flask. Add 2 c.c. of the digested mixture. Add about 50 c.c. of distilled water. Add 30 c.c. Nessler's reagent. Dilute to the mark and mix.

9. Compare the standard and unknown in the colorimeter, the standard being set at 20.

10. CALCULATION:

$$\frac{20}{R} \times 30 = \text{milligrams nonprotein nitrogen per 100 c.c. of blood.}$$

Use standard containing 0.3 milligram nitrogen made up to a total volume of 100 c.c. Set standard at 20. Make total volume of unknown blood filtrate up to 50 c.c.

The following table shows the nonprotein nitrogen in milligrams per 100 c.c. of blood corresponding to the different colorimeter readings with 5 c.c., 2 c.c. or 1 c.c. of blood filtrate for the test.

TABLE FOR NONPROTEIN NITROGEN IN BLOOD

Colorimeter Reading	Nonprotein Nitrogen in Milligrams per 100 c.c. Blood														
	Using 5 c.c. of Blood Filtrate					Using 2 c.c. of Blood Filtrate					Using 1 c.c. of Blood Filtrate				
	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
10	60.0	58 8	57.8	56.4	55.4	150.0	147.0	144.5	141.0	138.5	300	294	289	282	277
11	54.6	53.6	52.6	51.8	50.8	136.5	134.0	131.5	129.5	127.0	272	268	263	259	254
12	50.0	49.2	48.2	47.6	46.8	125.0	123.0	121.0	119.0	117.0	250	246	242	238	234
13	46.0	45.6	45.0	44.4	43.6	115.0	114.0	112.5	111.0	109.0	230	228	225	222	218
14	42.8	42.2	41.6	41.0	40.6	107.0	105.5	104.0	102.5	101.5	214	211	208	205	203
15	40.0	39.5	38.9	38.5	38.0	100.0	98.8	97.3	96.3	95.0	200	198	195	193	190
16	37.5	37.0	36.6	36.1	35.7	93.8	92.5	91.5	90.3	89.8	188	185	183	181	179
17	35.3	34.9	34.5	34.1	33.7	88.3	87.3	86.3	85.3	84.3	177	175	173	171	169
18	33.3	33.0	32.6	32.3	31.9	83.3	82.5	81.5	80.8	79.8	167	165	163	162	159
19	31.6	31.2	30.9	30.6	30.3	79.0	78.0	77.3	76.5	75.8	158	156	155	153	152
20	30.0	29.7	29.4	29.1	28.9	75.0	74.3	73.5	72.8	72.3	150	149	147	146	145
21	28.5	28.2	28.0	27.7	27.5	71.3	70.5	70.0	69.3	68.8	143	141	140	139	138
22	27.3	27.0	26.8	26.5	26.3	68.3	67.5	67.0	66.3	65.8	137	135	134	133	132
23	26.1	25.9	25.7	25.4	25.2	65.5	65.0	64.5	63.5	63.0	131	130	129	127	126
24	25.0	24.8	24.6	24.4	24.2	62.5	62.0	61.5	61.0	60.5	125	124	123	122	121
25	24.0	23.8	23.6	23.4	23.3	60.0	59.5	59.0	58.5	58.3	120	119	118	117	116
26	23.0	22.9	22.8	22.7	22.5	57.5	57.3	57.0	56.8	56.5	115	115	114	114	113
27	22.4	22.2	22.0	21.8	21.6	56.5	55.5	55.0	54.5	54.0	111	111	110	109	108
28	21.4	21.3	21.1	21.0	20.8	53.5	53.3	52.8	52.5	52.0	107	107	106	105	104
29	20.7	20.5	20.4	20.3	20.2	51.8	51.3	51.0	50.8	50.5	103	103	102	102	101

11. **Alternate Method.**—When using 5 c.c. of blood filtrate, set the unknown colorimeter cup at 15 millimeters and multiply the reading of the

standard cup by 2 which gives milligrams nonprotein nitrogen per 100 c.c. of blood.

NOTES.—1. In the digestion, bumping is often a source of serious difficulty. The most important cause of bumping lies in the condition of the test tube. In dry test tubes and beads the very fine pores are filled with air and until this air has been driven out by heat, localized formation of steam occurs and the boiling is smooth and even, but as these pores are gradually filled with the liquid bumping begins.

2. In case of bumping after repeated determinations, heat the tube to red heat in a flame, cool and rinse with alcohol.

3. The amount of nonprotein nitrogen in normal blood ranges from 25 to 40 milligrams per 100 c.c.

4. The nitrogen estimated by this method represents the nitrogen of blood constituents which are not thrown down by the precipitant but remain in solution. This nitrogen has been called the "nonprotein nitrogen" and "uncoagulable nitrogen." Of the total nitrogen of the blood, it is about 1 per cent. Its principally known constituents are urea, uric acid, creatinine, creatine and amino-acids. The nitrogen in these does not equal the total nonprotein nitrogen. The difference has been called the "undetermined nitrogen" and contains principally peptid and peptone nitrogen. Urea nitrogen represents from 40 to 65 per cent of the total, with a normal average of 50 per cent of the total nonprotein nitrogen. Findings higher than 40 milligrams indicate nitrogenous retention; the failure of the kidney to eliminate waste products. By determining the amounts of the known constituents, particularly urea, uric acid and creatinine, more detailed information is obtained than by the determination of the total nonprotein nitrogen only.

5. In the case of bloods containing excessive amounts of nonprotein nitrogen a cloudiness results, the determination should be repeated, using 2 or 1 c.c. portions of filtrate.

DETERMINATION OF AMINO-ACIDS

Principle.—The color developed by amino-acids in the presence of beta-naphthoquinone-sulphonic acid and alkali is compared with a standard solution of an amino-acid similarly treated.

Reagents.—1. STANDARD ACID GLYCINE SOLUTION (0.07 milligram of nitrogen per c.c.).—Make a stock solution of glycine (glycocoll) which will contain 0.1 milligram of nitrogen per c.c. by putting the following substances in a 500 c.c. volumetric flask:

> Glycine (pure)........ .... 268 mg.
> Sodium benzoate .... 1 gm.
> N/10 hydrochloric acid q. s. ad..... .... 500 c.c.

This stock solution seems to keep indefinitely.

The blood standard containing 0.07 milligram of nitrogen per c.c. is made by measuring 70 c.c. of the stock solution from a buret into a 100 c.c. volumetric flask and diluting to the mark with N/10 hydrochloric acid.

2. SPECIAL CARBONATE SOLUTION.—Fifty c.c. of saturated sodium carbonate solution are diluted to a volume of 500 c.c. Titrate 20 c.c. of N/10 hydrochloric acid with the sodium carbonate solution using methyl red as an indicator. Dilute so that 8.5 c.c. are equivalent to 20 c.c. of the acid. The carbonate solution is about 1 per cent.

3. SPECIAL ACETIC ACID—ACETATE SOLUTION.—Dilute 100 c.c. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate.

4. SODIUM THIOSULPHATE SOLUTION.—Four per cent solution of sodium thiosulphate.

Procedure.—1. Place 1 c.c. standard acid glycine in a test tube with a capacity of 30 to 35 c.c.

2. Add 3 c.c. water.

3. In similar test tube put 5 c.c. blood filtrate.

4. Add 1 drop of 0.25 per cent phenolphthalein solution to each tube.

5. Add 1 c.c. special carbonate solution to standard.

6. Add special carbonate solution to filtrate drop by drop until approximately the same shade of pink is reached (3 to 4 small drops are usually required).

7. Add 5 c.c. water to standard.

8. Prepare a fresh solution of sodium salt of beta-naphthoquinone-sulphonic acid (100 milligrams in 20 c.c. water).

9. Add 2 c.c. of this reagent to standard and 1 c.c. to filtrate.

10. Shake a little and let stand nineteen to thirty hours in a dark cupboard.

11. Add 2 c.c. of special acetic acid-acetate solution to standard. Add 1 c.c. to filtrate.

12. Add 2 c.c. thiosulphate solution to standard. Add 1 c.c. to filtrate.

13. Add 14 c.c. water to standard with blood pipet. Final volume equals 30 c.c. Add 7 c.c. water to filtrate. Final volume equals 15 c.c.

14. Mix and compare in colorimeter with standard set at 20.

15. CALCULATION:

$$\frac{20 \times 7}{R} = \text{milligrams amino-acid nitrogen per 100 c.c. blood}$$

16. The normal is 6 to 8 milligrams per 100 c.c. of blood.

DETERMINATION OF CREATININE

Principle.—The yellow-red color produced in a protein-free blood filtrate by the action of alkaline picrate is compared with the color similarly produced in a standard solution of creatinine.

Reagents.—1. STOCK CREATININE SOLUTION.—In a 100 c.c. volumetric flask dissolve 0.1 gram of creatinine in N/10 hydrochloric acid and dilute to the mark with same.

2. STANDARD CREATININE SOLUTION.—Pipet 3 c.c. of the stock creatinine solution into a 500 c.c. volumetric flask, add 100 c.c. of N/10 hydrochloric acid and dilute to mark with water (5 c.c. contain 0.03 milligram of creatinine).

3. HYDROCHLORIC ACID (approximately N/10).—Dilute 10 c.c. of hydrochloric acid to 1 liter.

4. SATURATED PICRIC ACID SOLUTION.—Place about 15 grams of purified picric acid in a large Erlenmeyer flask; add 1 liter of water; heat over low flame until the picric acid is dissolved. Cool and keep in dark. Decant the clear solution for use.

5. SODIUM HYDROXIDE (10 per cent solution).

6. ALKALINE PICRATE.—To 25 c.c. of the saturated picric acid solution add 5 c.c. of sodium hydroxide solution. This should be freshly prepared for each determination.

Procedure (Folin and Wu).—1. Pipet 10 c.c. of protein-free blood filtrate into a flask marked B.

2. Pipet 5 c.c. of the standard creatinine solution into a second flask, marked S, and add 15 c.c. of water.

3. Add 5 c.c. of the alkaline picrate solution to flask B, and 10 c.c. to flask S.

4. Mix each and let stand for ten minutes. Compare in the colorimeter.

5. CALCULATION:

$$S = \text{reading of standard}$$

$$R = \text{reading of blood filtrate}$$

$$x = \text{milligrams of creatinine per 100 c.c. of blood}$$

$$x = \frac{1.5S}{R} = \frac{30}{R} \text{ if standard is set at 20}$$

6. **Alternate Method.**—Set the cup of the unknown at 15 millimeters when the reading of the standard divided by 10 gives milligrams of creatinine per 100 c.c. of blood.

NOTES.—1. The normal range of creatinine is from 1 to 2 milligrams per 100 c.c. of blood.

2. Five c.c. of the standard give a color for accurate colorimetric comparison with filtrates whose creatinine content is not over 2 milligrams per 100 c.c. of blood. For bloods known or thought to contain values higher than this, use 10, 15, or 20 c.c. of the standard with proportionally less water. If the content be very high, use less filtrate with proper dilution. Before the addition of the alkaline picrate the volume of S should be 20 c.c. and the volume of the flask B should be 10 c.c.

3. The saturated picric acid solution should be made from purified picric acid as described below.

Benedict's Method for Purification of Picric Acid.—Heat 6 liters of water to boiling in a large porcelain enameled pail. Add 250 grams of anhydrous sodium carbonate. When dissolved, add gradually 500 grams of moist technical picric acid. Before all of the picric acid has dissolved, the mixture should be removed from the flame and stirred a few minutes until solution of the picric acid has been effected. Filtration is usually unnecessary. Allow the solution to stand for a few minutes. Decant it from the sediment, and allow to stand overnight at room temperature. Filter with suction, using a 23 centimeter hardened filter. Suck dry, wash with 2 liters of 10 per cent sodium chloride solution, and again suck as dry as possible. Turn off the suction. Pour 500 c.c. of diluted hydrochloric acid (1 part concentrated acid and 4 parts water) on the filter, and stir the mixture thoroughly with a porcelain spatula. This acid is then sucked through, and the process repeated with three more portions of the hydrochloric acid, a total of 2 liters. After the last portion of acid is sucked through, the picric acid on the filter is washed with 2 liters of cold distilled water and sucked dry. It is then removed from the filter and dried at about 90° C. and powdered. This product should read about 13.5 to 14 millimeters by the Folin-Doisy test.

4. The amount of creatinine is very constant for the individual and is not appreciably affected by diet, being almost entirely endogenous in origin. It is the last of the nitrogenous waste products to accumulate in nitrogenous retention. Hence its accumulation in the blood is of grave prognostic significance. In chronic conditions, once it begins to accumulate, it rarely decreases. A concentration of 5 milligrams or over per 100 c.c. of blood is usually followed by death within a short period. In acute conditions and acute exacerbations of chronic conditions, the accumulation decreases with the subsiding of the acute stage but when there has been a resulting damage to the kidney, the level will remain slightly above normal. The chief value of its determination is during the later stages of kidney disease.

DETERMINATION OF CREATINE

Principle.—When creatine is heated with dilute acids it is transferred into its anhydride, creatinine. The creatinine preformed and that formed from creatine are then determined together by treating with alkaline picrate, as in the determination of preformed creatinine. The creatine can be calculated when the preformed creatinine is known.

Reagents.—1. Creatinine Standard (5 c.c. equal 0.03 milligram).—See page 536.

2. Picric Acid (saturated solution).—See page 536.

3. Sodium Hydroxide (10 per cent).

4. Hydrochloric Acid (normal solution).—This may be prepared with sufficient accuracy by diluting 9 c.c. concentrated C.P. hydrochloric acid to 100 c.c. with distilled water.

Procedure (Folin and Wu).—1. Transfer 5 c.c. of protein-free blood filtrate to a test tube graduated at 25 c.c.

2. Add 1 c.c. of normal hydrochloric acid.

3. Cover the mouth of the test tube with tin foil and heat in the autoclave to 130° C. for twenty minutes.

4. Cool.

5. The standard is prepared as follows: Pipet 10 c.c. of the creatinine standard into a 50 c.c. volumetric flask. Add 2 c.c. of normal hydrochloric acid.

6. Add the alkaline picrate solution (*a*) 5 c.c. to the unknown, (*b*) 10 c.c. to the standard.

7. Let stand eight to ten minutes.

8. With distilled water dilute (*a*) the unknown to the 25 c.c. mark, (*b*) the standard to the 50 c.c. mark.

9. Compare in the colorimeter within fifteen minutes from the time the alkaline picrate was added.

10. Calculation with standard set at 20:

$$\frac{20}{R} \times 6 = \text{milligrams " total creatine " per 100 c.c. blood}$$

11. **Alternate Method.**—Set the cup of the unknown at 12 millimeters when one half of the reading of the standard will give creatine in milligrams per 100 c.c. of blood.

NOTES.—1. In the case of bloods containing large amounts of creatinine, 1, 2 or 3 c.c. of blood filtrate plus water enough to make approximately 5 c.c. are substituted for 5 c.c. of the undiluted filtrate.

2. The normal value for "total creatine" given by this method is about 6 milligrams per 100 c.c. blood.

DETERMINATION OF URIC ACID

Principle.—The color produced by the action of a phosphotungstic acid reagent with the uric acid in protein-free blood filtrate is compared with the color produced by the same reagent with a standard solution of uric acid.

Reagents.—1. STOCK URIC ACID SOLUTION.—Weigh on an analytical balance 1 gram of uric acid and transfer to a funnel on a 300 c.c. flask. Place 0.45 to 0.50 gram lithium carbonate in a beaker in about 150 c.c. of water and heat to 60° C., stirring until all the carbonate has dissolved. With the hot carbonate solution, rinse the uric acid into the flask and shake. As soon as a clear solution is obtained, cool under running water, with shaking, and transfer to a volumetric liter flask. Rinse and dilute to a volume of 400 to 500 c.c. Add 25 c.c. of formaldehyde, and after shaking to insure thorough mixing, add 3 c.c. glacial acetic acid. Shake to remove most of the carbonic acid and dilute to the mark. Keep in small, tightly stoppered bottles in a dark place.

2. STANDARD URIC ACID SOLUTION.—Dilute 5 c.c. of the stock solution in a liter volumetric flask to 800 c.c. with water; add 2 c.c. of formaldehyde and 20 c.c. of two-thirds normal sulphuric acid. Dilute to the mark (5 c.c. are equivalent to 0.025 milligram uric acid). This keeps about eight weeks.

3. SODIUM CYANIDE SOLUTION.—In a graduated, glass-stoppered cylinder, dissolve 10 grams of sodium cyanide; add 0.4 c.c. of ammonium hydroxide. Dilute to 200 c.c. Prepare fresh monthly.

4. URIC ACID REAGENT.—Place in a 1000 c.c. Erlenmeyer flask, 100 grams of sodium tungstate, 80 c.c. of phosphoric acid, and about 700 c.c. of water. Boil gently for two hours using a reflux condenser. Cool and dilute to 1 liter

Procedure (Brown).—1. Pipet 10 c.c. of protein-free blood filtrate into a 100 c.c. Erlenmeyer flask marked *B;* add 5 c.c. of water.

2. To two other flasks marked *SI* and *SII* add, respectively, 5 and 10 c.c. of the uric acid standard, and 10 and 5 c.c. of water.

3. To all flasks add, from buret, 5 c.c. of sodium cyanide solution. Then add 0.5 c.c. of the uric acid reagent.

4. Mix.

5. Allow to stand twenty minutes.

6. Compare in colorimeter the solution in flask *B* with the standard which it appears, by inspection, the more nearly to resemble in color depth.

7. CALCULATION:

$$SI = \text{reading of standard I}$$
$$SII = \text{reading of standard II}$$
$$R = \text{reading of blood filtrate}$$
$$x = \text{milligrams of uric acid in 100 c.c. of blood}$$
$$x = \frac{2 \ 5 \ SI}{R} \text{ or } x = \frac{5SII}{R}$$
$$x = \frac{50}{R} \text{ or } x = \frac{100}{R} \text{ if standard is set at 20}$$

8. **Alternate Method.**—When using standard I, set the cup of the unknown at 10 millimeters when the reading of the standard cup divided by 4 gives milligrams of uric acid per 100 c.c. of blood. If standard II is used divide by 2.

NOTES.—1. The normal range of uric acid is from 2 to 4 milligrams per 100 c.c. of blood.

2. The protein-free filtrate is prepared by the tungstic acid method of Folin and Wu as outlined previously.

3. Uric acid is the end-product of purin metabolism, and is partly exogenous and partly endogenous.

4. It is increased in kidney dysfunction but has no clinical value herewith which is not indicated by the urea nitrogen determination.

5. It is increased, with little or no increase in the other nitrogenous constituents in gout, and cardiac decompensation.

DETERMINATION OF BLOOD SUGAR

Principle.—Protein-free blood filtrate is heated with an alkaline cupric solution. The cuprous oxide precipitate formed by the glucose is dissolved by a phosphomolybdic acid solution. The resulting solution is compared colorimetrically with one similarly prepared from a standard glucose solution.

Reagents.—1. ALKALINE COPPER TARTRATE SOLUTION.—Dissolve 40 grams of pure anhydrous sodium carbonate in about 400 c.c. of distilled water in a liter flask. Add 7.5 grams of tartaric acid, and when this is dissolved add 4.5 grams of crystallized copper sulphate. Mix and make up to a volume of 1 liter.

Test for the absence of cuprous copper by transferring 2 c.c. to a test tube and adding 2 c c of the molybdate phosphate solution; the deep blue of the copper should almost completely vanish.

If the chemicals used are not pure, a sediment of cuprous oxide may form in the course of one or two weeks. If this happens, remove the clear supernatant reagent or filter through a good quality filter paper. Test for copper. This reagent keeps indefinitely.

2. MOLYBDATE-PHOSPHATE SOLUTION. — Transfer 70 grams of molybdic acid (C.P. Baker's analyzed "special") to a liter beaker. Add 10 grams of sodium tungstate, 400 c.c. of 10 per cent sodium hydroxide and 400 c.c. of distilled water. Boil vigorously for twenty to forty minutes (to remove ammonia present in molybdic acid). Cool and dilute to about 700 c.c. Add 250 c.c. of concentrated (85 per cent) phosphoric acid. Dilute to 1000 c.c.

FIG 285 — FOLIN BLOOD SUGAR TUBE

The solution should be water clear. If it has a yellow tinge, the chemicals used are not pure enough and there will be an error in the determination.

3. BENZOIC ACID.—Dissolve 2.5 grams of benzoic acid in 1 liter of hot water and cool. Transfer to a bottle; the solution will keep indefinitely. Filter as necessary.

4. STANDARD SUGAR SOLUTIONS.—*A. Stock* —Weigh 1 gram of pure dextrose (glucose) on an analytical balance and dissolve in about 50 c.c. of the benzoic acid solution. Transfer to a 100 c.c. volumetric flask, rinse, and fill to the mark with benzoic acid solution. This 1 per cent standard stock solution keeps indefinitely.

B Standard Containing 10 Milligrams Dextrose per 100 C.C.—Pipet 5 c.c. of stock solution into a 500 c.c. volumetric flask and dilute to the mark with benzoic acid solution.

C. Standard Containing 20 Milligrams Dextrose per 100 C.C.—Pipet 10 c.c. of stock solution into a 500 c.c. volumetric flask and dilute to the mark with benzoic acid solution.

Diluted standards *B* and *C* should be made fresh every month.

Procedure (Folin and Wu).—1. Pipet 2 c.c. of tungstic acid blood filtrate into a special Folin sugar tube (Fig. 285).

2. To a similar tube, add 2 c.c. of standard sugar solution B containing 10 milligrams dextrose per 100 c.c., and to a third tube add 2 c.c. of standard C containing 20 milligrams dextrose per 100 c.c.

3. To each tube add 2 c.c. of the alkaline copper tartrate solution. (The surfaces of the mixtures must now be in the constricted part of the tubes.)

4. Transfer the tubes to a boiling water bath and heat for six minutes.

5. Cool for two to three minutes in a cold water bath without shaking.

6. Add to each tube 2 c.c. of the molybdate phosphate solution.

7. Let stand for three minutes; dilute the resulting solution to the 25 c.c. mark.

8. Insert a rubber stopper and mix. (It is essential that adequate attention be given to the mixture because the greater part of the blue color is formed in the bulb of the tube.)

9. Compare in a colorimeter with the nearest matching standard set at 20. The two standards are adequate for practically all cases, as they cover a range from about 70 to nearly 400 milligrams of dextrose per 100 c.c. of blood. If colorimeter reads below 13.3 with SI, SII should be used. If colorimeter reads above 26.7 with SII, SI should be used.

10. CALCULATION:

$$\frac{20}{R} \times 100 = \text{milligrams dextrose per 100 c.c. of blood when the standard}$$
containing 10 milligrams per 100 c.c. is used.

$$\frac{20}{R} \times 200 = \text{milligrams dextrose per 100 c.c. of blood when the standard}$$
containing 20 milligrams per 100 c.c. is used

The table on page 542 shows the glucose in milligrams per 100 c.c. blood corresponding to colorimeter readings using the two different standards.

Alternate Method.—When using standard No. 1, set the cup of the unknown at 20 millimeters. When the reading of the standard multiplied by 5 equals milligrams of sugar per 100 c.c. of blood. When using standard No. 2, set the cup of the unknown at 10 millimeters when the reading of the standard multiplied by 20 equals milligrams of sugar per 100 c.c. of blood.

NOTES.—1. Cooling of the alkaline cuprous oxide suspension before adding the phosphate molybdate solution is not essential, and in case of one or two determinations only may be omitted. In a large series of determinations it is probably best to use it. The important point is that the standard and the unknown should not only be heated the same length of time but should also have substantially the same temperature when the acid reagent is added.

2. The normal range of concentration of blood sugar for the fasting adult is from 80 to 110 milligrams per 100 c.c. of blood.

3. The protein-free filtrate for this method is prepared by the tungstic acid method of Folin and Wu, as outlined in previous method.

TABLE OF BLOOD SUGAR VALUES

Color-imeter Reading	Glucose in Milligrams per 100 c.c. Blood									
	Standard No. 1 (Containing 10 Milligrams Glucose in 100 c.c.)					Standard No. 2 (Containing 20 Milligrams Glucose in 100 c c.)				
	0 0	0 2	0 4	0 6	0 8	0 0	0.2	0 4	0 6	0 8
5	400	385	370	357	345	800	769	740	714	689
6	333	323	313	303	294	667	645	625	606	588
7	286	278	270	263	256	571	555	540	526	512
8	250	244	238	233	227	500	487	476	465	454
9	222	217	213	209	204	444	434	425	417	408
10	200	196	192	189	185	400	392	384	377	370
11	182	179	175	172	169	363	357	350	344	338
12	167	164	161	159	156	333	327	322	317	312
13	154	152	149	147	145	307	303	298	295	290
14	143	141	139	137	135	286	282	278	273	270
15	133	132	130	128	127	267	263	260	256	254
16	125	124	122	121	119	250	247	244	241	238
17	118	116	115	114	112	236	233	230	227	224
18	111	110	109	108	106	222	220	218	215	212
19	105	104	103	102	101	211	209	206	204	202
20	100	99	98	97	96	200	198	196	194	192
21	95	94	93	93	91	189	189	187	185	183
22	91	90	89	88	87	182	180	179	177	175
23	87	86	86	85	84	173	172	172	169	168
24	83	83	82	81	81	167	165	164	163	161
25	80	79	79	78	78	160	159	158	156	155
26	77	76	76	75	75	154	153	152	150	149
27	74	74	73	73	72	147	147	146	145	144
28	71	71	71	70	69	142	142	140	140	139
29	69	69	68	68	67	137	137	136	135	134

4. If the blood filtrate gives a color too deep for accurate colorimetric comparison with the stronger standard, the test is repeated, substituting for the 2 c.c. of blood filtrate in tube B, 1 c.c. of blood filtrate and 1 c.c. of distilled water. The final result must therefore be doubled.

5. Dextrose determinations are made immediately after taking the blood sample as the dextrose rapidly disappears by glycolysis. Efficient refrigeration retards, but does not prevent, glycolysis.

6. When the analysis cannot be made immediately, the proteins of the blood should be precipitated and the filtrate, to which are added a few drops of toluene, placed in the refrigerator. This filtrate will give accurate readings for twenty-four hours after precipitation.

7. A rise in blood sugar follows absorption from the intestinal tract. The fasting level is again reached within three hours after the ingestion of food. In some pathologic conditions, e.g., diabetes mellitus, this return is delayed

and the rise is higher than in normals. This forms the basis of sugar tolerance tests.

8. Hyperglycemia is found in diabetes mellitus, some cases of advanced nephritis, and frequently in emotional states.

9. Hypoglycemia has been reported after thyroidectomy and in some hypo-endocrine conditions.

10. One determination of the blood sugar is not sufficient on which to base a diagnosis of hyperglycemia; the high level must be constant.

11. In "renal glycosuria" there is no hyperglycemia although there is glycosuria.

12. Glycosuria (the presence of glucose in the urine when examined by usual laboratory tests) is not dependent directly on the level of the blood sugar. The point of concentration which when reached results in glycosuria has been called the "renal threshold." This appears to be individual and not a definite point; for most normal persons concentrations of 160 to 180 milligrams result in glycosuria. These figures are higher in diabetes mellitus and late nephritis, but lower in "renal glycosuria." The significance of glycosuria cannot be determined without simultaneous blood sugar determinations.

MICROMETHOD FOR THE DETERMINATION OF SUGAR

Principle.—When a glucose solution is heated with an alkaline ferricyanide solution, the ferricyanide is reduced to ferrocyanide. A blue color is produced when ferrocyanide reacts with a ferric iron solution. The glucose in a protein-free filtrate is determined by comparing it with a standard glucose solution.

Reagents.—1. TUNGSTIC ACID SOLUTION.—Transfer 20 c.c. of 10 per cent sodium tungstate to a volumetric liter flask. Dilute to a volume of 700 c.c.; add, with shaking, 160 c.c. of N/12 sulphuric acid and dilute with water to 1000 c.c.

2. POTASSIUM FERRICYANIDE SOLUTION.—Dissolve 2 grams of C.P. potassium ferricyanide in distilled water and dilute to a volume of 500 c.c. The major part of this solution should be kept in a brown bottle in a dark closet. The reagent in daily use should also be kept in a brown bottle.

3. SODIUM CYANIDE-CARBONATE SOLUTION.—Transfer 8 grams of anhydrous sodium carbonate to a 500 c.c. volumetric flask. Add 40 to 50 c.c. of water and shake, to promote rapid solution. Add 150 c.c. of freshly prepared 1 per cent sodium cyanide solution; dilute to volume and mix.

4. FERRIC IRON SOLUTION.—Fill a liter cylinder with water. Suspend on a copper wire screen, just below the surface, 20 grams of soluble gum ghatti, and leave overnight (eighteen hours). Remove the screen, and strain the liquid through a double layer of a clean towel. Add to this extract a solution of 5 grams of anhydrous ferric sulphate in 75 c.c. of 85 per cent phosphoric acid plus 100 c.c. of water. Add to the mixture, a little at a time, about 15 c.c.

of 1 per cent potassium permanganate solution to destroy certain reducing materials present in gum ghatti. The slight turbidity of the solution will disappear completely, if kept at 37° C. for a few days.

5. STANDARD GLUCOSE SOLUTION.—To a liter volumetric flask add 1 c.c. of stock (1 per cent) glucose solution (see page 540); add 125 c.c. of 0 2 per cent benzoic acid; dilute to mark and mix. One c.c. = 0 01 milligram of glucose

6. PICRATE LIGHT FILTER.—Dissolve 5 grams of picric acid in 100 c.c. of methyl alcohol and add 5 c.c. of 10 per cent sodium hydroxide solution. Place a pack of eight to ten filter papers (of the correct size to cover colorimeter lamp) on a level and smooth mat of newspapers. Pour the acid picrate solution onto the filters until the papers are saturated and an excess of solution which filters through at the bottom and flows out a distance of at least 2 centimeters on the newspaper mat. When all the liquid has evaporated and the filter papers are perfectly dry, pour over the pack an excess of a 3 per cent solution of paraffin in benzine (gasoline) and again leave the papers to dry. A heavy filter with good absorbing qualities is best.

Test of Adequacy of Light Filter —Half fill one colorimeter cup with 0.2 per cent potassium ferricyanide solution and half fill the other cup with water. Set both plungers at 20 millimeters, and make sure that there are no air bubbles under the plungers. With the light filter in place, adjust the colorimeter so that the two fields look alike. If the light filter is inadequate, the equality of the two fields cannot be obtained.

Procedure (Folin).—1. With the special 0.1 c.c. blood pipet collect 0.1 c.c. of blood from an ear or finger prick. Transfer it to 10 c.c. of dilute tungstic acid in a centrifuge tube.

2. Stir well and centrifuge.

3. Transfer 4 c.c. of the water-clear supernatant extract to a test tube graduated (with a ring going all around) at 25 c.c. Transfer 4 c.c. of the standard sugar solution to another similar tube. To each tube add 2 c.c. of the 0.4 per cent potassium ferricyanide solution and 1 c.c. of the cyanide-carbonate solution.

4. Heat in boiling water for eight minutes and cool in running water for one to two minutes.

5. Add 5 c.c of the ferric iron solution and mix.

6. Let stand for one to two minutes and then dilute to the 25 c.c. mark. If foam obscures the mark, add one to two drops of alcohol.

7. Half fill the colorimeter cups with the green-colored standard, set the two plungers at a height of 20 millimeters, and cover the opening of the light box with the picric acid light filter. Adjust the position of the colorimeter and of the mirror glass reflector until the two fields look exactly alike. Replace the standard in the left cup with the unknown solution. Compare the two solutions.

Colorimeter readings between 40 and 5 millimeters may be accepted as correct.

8. CALCULATIONS:

$$x = \text{milligrams of glucose in 100 c.c. blood}$$

$$S = \text{reading of the standard}$$

Set the unknown at 20 on the left.

$$x = 5S$$

In hyperglycemic blood, set the unknown at 10 on the left.

$$x = 10S$$

NOTES.—1. This method is recommended for use in cases where it is difficult to do a venipuncture or when the patient must be bled frequently.

2. Blood taken from the finger is a mixture of venous and arterial blood and the sugar values after a glucose meal are higher than corresponding ones from venous blood alone.

3. The picrate filters are to eliminate the yellow color of the ferricyanide solution. When the colors are nearly the same, the filter may be dispensed with.

DETERMINATION OF SUGAR TOLERANCE

1. Instruct the patient to take a breakfast of one or two eggs, two slices of bread with butter and a cup of weak tea or coffee. No other food is taken until the tests are completed. Or breakfast may be omitted and the test conducted after fasting overnight (preferred).

2. Three to four hours later urine (No. 1) and blood (No. 1) are taken for sugar determinations.

3. Immediately thereafter give 1.75 grams of glucose per kilogram of body weight dissolved in 500 c.c. of water; flavor with lemon juice and cool with ice.

4. One hour later take urine (No. 2) and blood (No. 2) for sugar determinations.

5. One hour later (two hours after taking the glucose), take urine (No. 3) and blood (No. 3).

6. One hour later (three hours after taking the glucose), take urine (No. 4) and blood (No. 4).

7. As a general rule these are sufficient although it is sometimes advisable to take another specimen of urine (No. 5) and blood (No. 5) again one hour later (four hours after taking the glucose).

8. Conduct sugar determinations on all samples of blood and urine. Plot the blood sugar determinations in a curve.

9. Normally the blood sugar rises to about 160 milligrams per 100 c c. with none or but a trace of sugar in the urine at the end of the first hour, reaching the normal fasting level at the end of two to three hours.

10. In diabetes mellitus the blood sugar rises above 170 milligrams per 100

c.c. with sugar in the urine and does not reach the fasting level until after three or four hours.

11. In "renal glycosuria" the blood sugar rises with large amounts in the urine but declines rapidly, reaching the fasting level in three hours

12. A sugar tolerance test should not be conducted if diabetes mellitus in an advanced state is known to be present.

DETERMINATION OF ACETONE BODIES

Principle.—Acetone, aceto-acetic acid, and oxybutyric acid are all determined as acetone, the first two by distillation with acid, the last, by distillation with sulphuric acid and potassium bichromate. Acetone in the distillate is determined by means of its color reaction with salicylic aldehyde in alkaline solution, which is due to formation of dihydroxydibenzene acetone.

Reagents.—1. SODIUM HYDROXIDE SOLUTION (32 per cent).

2. SALICYLIC ALDEHYDE.—Eimer and Amend's "Acid Salicylous, synthetic (salicylic aldehyde)" and Kahlbaum's technical salicylaldehyde were found more satisfactory than other preparations by the authors of the method.

3. SULPHURIC ACID (50 per cent).

4. POTASSIUM BICHROMATE SOLUTION (0.2 per cent).

5. SODIUM TUNGSTATE SOLUTION (10 per cent).

6. SULPHURIC ACID (two-thirds normal).

7. STOCK SOLUTION OF ACETONE.—Prepare a solution containing 1 c.c. of acetone in a liter of water. Determine the acetone content of this solution by iodine titration, as follows: Measure 25 c.c. of normal potassium hydroxide into a 250 c.c. flask. Add 10 c.c. of the above solution of acetone. Mix, stopper, and allow to stand for five minutes. Add, drop by drop, 20 c.c. of N/10 iodine solution, shaking the mixture constantly. Stopper and allow to stand for fifteen minutes. Add 25 c.c. of normal sulphuric acid (sufficient to neutralize the potassium hydroxide) and titrate the excess of iodine with N/10 thiosulphate. One c.c. of N/10 iodine equals 0 967 milligram of acetone. With the above amounts:

c.c. of N/10 iodine—c.c. N/10 thiosulphate×0.0967 = milligrams of iodine
per c.c. of solution

Dilute the solution so that 1 c.c. equals 0.1 milligram of acetone. This stock solution can be kept for about a month without deterioration.

8. STANDARD SOLUTION.—By 1 : 10 dilution of the stock solution a standard solution containing 0.01 milligram of acetone per c c. is prepared for use in the actual determination. It is best to make this dilute solution every second day, and keep it in a glass-stoppered container when not in use.

Procedure (Behre and Benedict).—1. PREFORMED ACETONE AND ACETONE FROM ACETO-ACETIC ACID.—The blood proteins are precipitated by the regular

Folin-Wu method, making a dilution of the blood of 1 : 10. From 10 to 100 c.c. of the filtrate, depending on the acetone content, are transferred to a 300 c c. distilling flask; 3 or 4 drops of concentrated sulphuric acid, diluted 1 : 1, added, and the volume made up to 50 to 75 c.c., if the smaller volumes of filtrate are used.

2. The flask is tightly fitted with a cork stopper and connected with a water-cooled condenser (Fig. 286). The condenser is provided with a bent glass tube, which has been drawn out long enough and to a sufficiently small diameter to reach to the bottom of a volumetric flask or graduated test tube, and which dips below the surface of a minimum amount of water in the flask. None of the connections should be of rubber. Rubber stoppers covered with tin foil, or cork stoppers, often renewed, can be used. The pre-formed acetone and acetone from aceto-acetic acid are then distilled. The distillation is made into a 20 c.c. receiving flask or graduated test tube, unless the acetone content of the amount of blood filtrate used is known to be high (above 0.05 milligram), in which case the distillation is made into a 25 c.c. flask or graduated tube. If the amount of acetone in the filtrate used is known to be o 1 milligram or more, the distillation may be made to 50 c.c. In any case the distillation is stopped just before the desired volume has been reached, and the distillate is made up to volume.

FIG 286.—DIGESTION APPA-
RATUS FOR TOTAL ACETONE
BODIES

3. Standards are prepared in test tubes, by using from 0.5 to 5.0 c.c. of the dilute (0.01 milligram per c.c.) acetone solution, water being added in each case to make the volume 5 c c Unless the approximate acetone content of the unknown solution is known, standards containing 0.005, 0.01, 0.02, 0.03, and 0.05 milligram had best be made. Five c.c. of the distillate and the 5c.c. standards are then treated uniformly. To each add exactly 5 c c. of 32 per cent sodium hydroxide and 10 drops of salicylic aldehyde. The contents of the tubes are mixed by side-to-side shaking, and the tubes then immersed in a boiling water bath for from three to five minutes. If the salicylic aldehyde does not dissolve easily, the tubes must be shaken until solution is effected. After the heating the tubes are removed and allowed to cool. The solutions are filtered, and colorimetric comparison is made. The standard used should be of such a concentration that the unknown solution gives a reading between 11 and 19 millimeters with the standard set at 15 millimeters.

4. CALCULATION:

$$\frac{x}{R} \times \frac{P}{S} \times \frac{T}{5} \times 100 = \text{number of grams of acetone in 100 c.c. of blood}$$

$x = $ reading of standard

$R = $ reading of unknown

$P = $ grams acetone in standard

$S = $ c c of blood used (one-tenth the volume of blood filtrate used)

$T = $ c.c. volume of total distillate

ACETONE FROM BETAHYDROXYBUTRIC ACID —1. After the distillation of the preformed acetone, a 100 c c. receiving flask is substituted for the 20 c.c. flask, the residue in the distilling flask is brought to a boil, and 30 c.c. of half concentrated sulphuric acid and 20 c.c. of 0.2 per cent potassium bichromate are added gradually through the dropping funnel while a slow distillation goes on. Fifty c.c. more of the bichromate are added after ten minutes, and 50 c.c. more after another interval of ten minutes. The distillation should occupy at least thirty minutes, and the volume of distillate is kept down to 100 c.c.

2. When the distillation to 100 c.c. is almost complete, the receiving apparatus is again disconnected, the bent tube washed down with a little water, and the distillate made up to 100 c.c., and mixed.

3. Acetone is determined colorimetrically in 5 c.c. of the distillate as described above, the same calculations being used. The above directions apply if the actual volume of filtrate used is known to contain 0.1 milligram of betahydroxybutyric acid (as acetone) or more. If it is expected to contain less than 0.1 milligram, the distillation is carried out without regard to the volume of distillate collected, and after the thirty-minute period the distillate is redistilled into a 20, 25, or 30 c.c. volumetric flask or graduated test tube, according to the amount of acetone expected. The distillate is made up to volume and the acetone content of 5 c c. of the distillate determined as described above. The formula given applies for all calculations.

DETERMINATION OF THE CARBON DIOXIDE CAPACITY OF BLOOD PLASMA

Principle.—Blood plasma is shaken in a separatory funnel filled with an air mixture the carbon dioxide tension of which approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known volume of the saturated plasma is then run into a special apparatus, acid is added and carbon dioxide is liberated by the production of a partial vacuum. The carbon dioxide is measured at atmospheric pressure and the volume corresponding to 100 c.c. plasma is calculated

Collection of Blood.—For at least an hour before the blood is drawn the subject should avoid vigorous muscular exertion, as this, presumably because

of the lactic acid formed, lowers the bicarbonate of the blood. About 8 c.c. of blood from an arm vein are aspirated directly into an oxalated test tube (Fig. 282) carrying enough paraffin oil to make a column 1 centimeter high.

It is essential that the blood be collected with minimum gain or loss of carbon dioxide, as hydrochloric acid is transferred from plasma to cells by increase of free carbon dioxide in the former, and vice versa, with resultant change of not only free carbon dioxide, but also of bicarbonate in the plasma. Consequently, overaccumulation of carbon dioxide in the venous blood is avoided by using as little stasis as possible. When stasis is necessary, the ligature is released as soon as the vein is entered and a few seconds allowed for the stagnant blood to flow out before the main sample is drawn. It is equally necessary to avoid loss of carbon dioxide while the plasma is still in contact with the corpuscles *in vitro*. In order to prevent such loss, the blood may be drawn into a tube arranged for this purpose. After the sample has been drawn the stopper is loosened and the blood stirred with the inlet tube in order to assure distribution of the oxalate. The tube should not be shaken or inverted. The blood is centrifuged within a half hour and the plasma drawn off. In place of the tube, a syringe may be used, if the blood is drawn with minimum suction and ejected under oil into a centrifuge tube.

In case the carbon dioxide capacity cannot be determined at once, the plasma is transferred to a paraffin-lined tube, covered with oil, stoppered and placed on ice where it will keep for a few days. Plasma in ordinary glass can be kept for only a few hours as sufficient alkali dissolves in longer intervals to measurably increase the carbon dioxide capacity.

Saturation with Carbon Dioxide.—After centrifugation, the plasma (3 c.c. or more if there is plenty of material) which should be at room temperature, is placed in a separatory funnel of about 300 c.c. capacity and the funnel is filled with alveolar air from the lungs of the operator. The air is passed through a bottle full of glass beads before it enters the funnel in order to bring the moisture content down to saturation at room temperature. If one blows directly into the separatory funnel, enough moisture condenses on the walls to dilute the plasma appreciably. The operator at the end of an ordinary inspiration expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel is inserted just before the expiration is finished so that there is no opportunity for air to be drawn back into the funnel. Repeat three times. The funnel is then rotated for two minutes in such a manner that the plasma is distributed as completely as possible about the walls, forming a thin layer, which quickly approaches equilibrium with the carbon dioxide in the air.

Reagents.—1. AMMONIA SOLUTION (1 per cent by volume, C.P.).—Add a few drops of phenolphthalein to color. This solution is advantageously kept in a bottle above the apparatus so that it can be introduced into the cup through a rubber tube which carries a pinch clamp.

2. SULPHURIC ACID SOLUTION (5 per cent C.P.).

3. CAPRYLIC ALCOHOL (pure; also known as "octyl alcohol, secondary").

4. MERCURY (metallic and purified).

Procedure (Van Slyke and Cullen).—The analysis is performed in a Van Slyke blood gas apparatus, preferably the precision model with Shohl trap and water jacket (Fig. 287), mounted on a board which can be mechanically shaken by a motor.

1. The apparatus should have been previously rinsed out with 1 per cent ammonia water followed by three rinsings with distilled water.
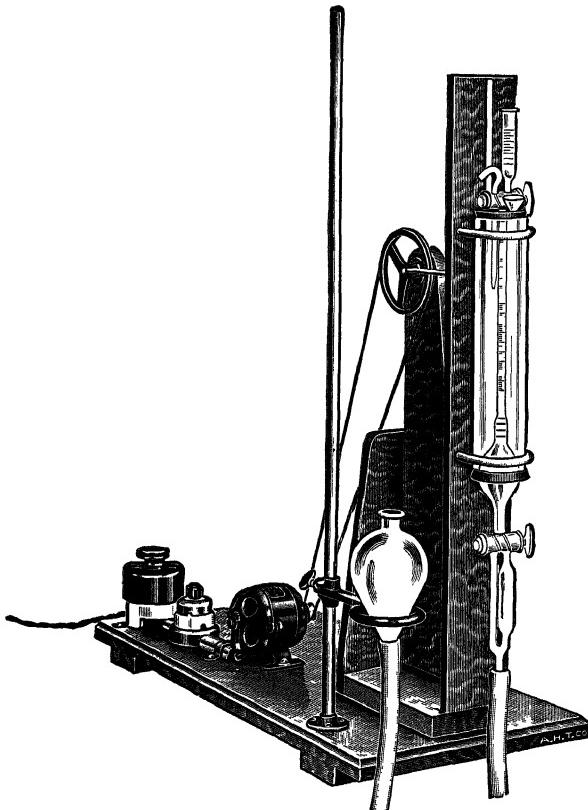
FIG 287—PRECISION MODEL OF THE VAN SLYKE GAS ANALYSIS APPARATUS WITH WATER JACKET AND STATIC SHAKER.

2. Before beginning a determination it is necessary to test the apparatus for gas leakage. The entire apparatus, including the capillaries of the upper stopcock, is filled with mercury and the mercury bulb lowered so that a vacuum is obtained, the mercury falling below the lower stopcock. The leveling bulb is then raised again. If the apparatus is tight, the mercury will refill it completely and strike the upper stopcock with a sharp click.

3. The mercury leveling bulb is placed about on a level with the lower stopcock. With an Ostwald-Folin pipet, introduce 1 c c. of the saturated

plasma in the cup, express the last drop by closing the pipet with one finger and warming the bulb of the pipet with the palm of the hand, and quickly let most of it run down into the upper stem of the apparatus.

4. The cup is now washed with two portions of about 0.5 c.c. each of water, care being taken that no air enters the apparatus with the liquid.

5. Two small drops of caprylic alcohol are next added to the cup from a capillary pipet and permitted to flow into the capillary.

6. About 1 c c. of 10 per cent lactic acid is poured into the cup. Enough of the acid is admitted (about 0.5 c.c.) into the 50 c.c. chamber, carrying the caprylic alcohol along with it, so that the total volume of water in the apparatus is exactly 2.5 c.c.

7. A little mercury is dropped into the cup and allowed to flow down to the upper stopcock in order to make a seal.

8. The leveling bulb is lowered and the mercury in the apparatus is allowed to fall until the mercury meniscus has dropped to the 50 c.c. mark; the lower stopcock is then closed.

9. Shake for nearly two minutes.

10. Read the barometer and take the temperature.

11. Run almost all of the fluid into the right lower collecting chamber and trap it there.

12. Readmit mercury through the left channel to the 50 c.c. chamber.

13. The gas is brought to atmospheric pressure by raising the leveling bulb beside the pipet so that the mercury levels are the same. The side arm on the leveling bulb facilitates this. For strict accuracy the mercury meniscus in the side arm should be raised above the level of the mercury meniscus in the apparatus by a distance equal to one-fourteenth of the height of the water column.

14. The volume of gas above the short column of water is read at once.

15. After the determination has been finished, the water solution is forced out together with a little mercury, and the apparatus is washed with 1 per cent ammonia solution and distilled water.

16. Repeat the determination after resaturating the plasma with alveolar air.

17. CALCULATIONS: The calculation of the carbon dioxide capacity is made with the aid of a table which contains corrections for the air (about 0.05 c.c.) dissolved by the 2.5 c.c. of water introduced into the apparatus, for the carbon dioxide physically dissolved in 1 c.c. plasma (about 0.5 c.c.), for the carbon dioxide not removed from the solution by one extraction (4 to 5 per cent), for the carbon dioxide reabsorbed by water (factor 1.017), and corrections for temperature, pressure and water vapor, so that the gas volume is reduced to standard conditions of 0° C. and 760 millimeters pressure.

The volume of gas observed is multiplied by the factor $B/760$, B being the barometric reading. The factor $B/760$ can be ascertained by referring to the table. The volume of gas observed is multiplied by this and the figure

obtained is located in the first column headed "Observed Volume of Gas $\times$ $B/760$." The line is carried across horizontally to the column which is headed by the temperature at which the reading was made, and the result read off.

The results of the carbon dioxide capacity determination are expressed in "volumes per cent" and have reference to the number of c.c. of carbon dioxide measured at 0° C. and 760 millimeters pressure, chemically bound as bicarbonate in 100 c.c. of blood plasma which has been equilibrated at the carbon dioxide tension of normal arterial blood.

NOTES.—1. Normal range: In a normal resting adult the range is from 53 to 77 volumes per cent, and for normal infants about 10 volumes per cent lower. A result of from 53 to 40 volumes per cent shows a mild acidosis, generally without visible symptoms. In cases yielding from 40 to 31 volumes per cent symptoms may be apparent. Less than 31 volumes per cent indicates a severe acidosis.

2. When not in use, the pipet should be kept filled with distilled water.

3. Free carbonic acid is present in the body fluids in such concentration that it binds as bicarbonate all bases not bound by other acids; it therefore represents the excess of base which is left after all the nonvolatile acids have been neutralized and is available for neutralization of further acids. In this sense the bicarbonate constitutes the alkaline reserve of the body. Entrance of free acids reduces it to an extent proportional to the amount of the invading acids. Both in normal and pathologic metabolism, acids invade the blood and bind some of the alkali. Normally the kidneys are able to eliminate these acids while retaining the alkali; by this mechanism the body is able to excrete an acid urine from an alkaline blood.

4. Acidosis is a condition caused by acid retention sufficient to lower either the bicarbonate below normal or the P^H of the blood below normal (*i.e.,* toward the acid side). In diabetic acidosis, the acid-base balance is disturbed by abnormal formation of nonvolatile acids (*e.g.,* aceto-acetic and beta-oxybutyric acids), while in nephritis, it is due to the failure of the kidney mechanism of elimination. In both cases the available alkali is bound by these acids reducing it in proportion to the amount of acid. Under nearly all circumstances in which the respiratory apparatus is not specifically affected, the quantity of carbonic acid is so regulated that a normal P^H is maintained.

5. This method suffices for the study of such metabolic conditions as diabetes, nephritis, and marasmus, in which the acid-base disturbance is due to retention of nonvolatile acids while the respiratory control of the blood reaction is unaffected. This method is not adequate to cover conditions in which the respiratory control is so disturbed that the P^H becomes abnormal, *e.g.,* in anesthesia.

6. In such cases a determination of the hydrogen ion concentration together with the carbon dioxide content of the venous blood is of greater advantage in determining the source of the disturbance of the acid-base balance.

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA

Observed Volume of Gas $\times B/760$	C.C. of Carbon Dioxide Reduced to 0° 760 mm. Bound as Bicarbonate by 100 c.c. of Plasma				Observed Volume of Gas $\times B/760$	C.C. of Carbon Dioxide Reduced to 0° 760 mm. Bound as Bicarbonate by 100 c.c. of Plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.3	10.1	10.9	12.0					
0.21	10.3	11.1	11.9	12.8	0.61	49.5	49.8	50.2	50.3
0.22	11.2	12.0	12.8	13.7	0.62	50.5	50.8	51.2	51.3
0.23	12.2	13.0	13.8	14.5	0.63	51.5	51.8	52.2	52.3
0.24	13.2	13.9	14.7	15.4	0.64	52.5	52.8	53.1	53.2
0.25	14.1	14.9	15.7	16.4	0.65	53.5	53.7	54.1	54.1
0.26	15.1	16.0	16.7	17.3	0.66	54.5	54.7	55.0	55.0
0.27	16.2	16.9	17.7	18.3	0.67	55.5	55.7	56.0	56.0
0.28	17.1	17.9	18.6	19.2	0.68	56.5	56.6	56.9	56.9
0.29	18.1	18.8	19.5	20.1	0.69	57.5	57.7	58.0	57.9
0.30	19.1	19.8	20.5	21.2	0.70	58.4	58.6	58.9	58.9
0.31	20.0	20.8	21.5	22.1	0.71	59.4	59.6	59.9	59.8
0.32	21.0	21.8	22.5	23.0	0.72	60.4	60.5	60.8	60.7
0.33	22.1	22.7	23.4	23.9	0.73	61.3	61.5	61.7	61.6
0.34	23.0	23.7	24.4	24.9	0.74	62.3	62.4	62.7	62.6
0.35	24.0	24.6	25.3	25.8	0.75	63.3	63.4	63.6	63.5
0.36	25.0	25.6	26.2	26.7	0.76	64.2	64.3	64.7	64.5
0.37	25.9	26.6	27.1	27.7	0.77	65.2	65.4	65.6	65.4
0.38	27.0	27.5	28.2	28.6	0.78	66.2	66.4	66.6	66.4
0.39	28.0	28.6	29.2	29.6	0.79	67.2	67.3	67.5	67.3
0.40	28.8	29.5	30.1	30.5	0.80	68.2	68.3	68.4	68.2
0.41	29.9	30.5	31.0	31.5	0.81	69.2	69.2	69.4	69.1
0.42	30.8	31.4	32.0	32.4	0.82	70.1	70.2	70.3	70.2
0.43	31.8	32.4	33.0	33.4	0.83	71.1	71.1	71.3	71.1
0.44	32.8	33.4	34.0	34.4	0.84	72.2	72.2	72.3	72.0
0.45	33.8	34.4	34.9	35.3	0.85	73.0	73.2	73.3	73.0
0.46	34.8	35.3	35.9	36.2	0.86	74.1	74.1	74.2	73.8
0.47	35.8	36.3	36.8	37.1	0.87	75.1	75.0	75.2	74.8
0.48	36.7	37.2	37.8	38.0	0.88	76.0	76.0	76.1	75.7
0.49	37.7	38.2	38.7	39.0	0.89	77.1	77.0	77.0	76.6
0.50	38.7	39.2	39.6	40.0	0.90	78.1	78.0	78.1	77.6
0.51	39.8	40.2	40.6	41.0	0.91	79.1	79.0	79.0	78.6
0.52	40.7	41.1	41.6	41.9	0.92	80.0	80.1	80.0	79.5
0.53	41.7	42.1	42.6	42.8	0.93	81.0	81.0	81.0	80.5
0.54	42.7	43.1	43.5	43.7	0.94	82.0	81.8	82.0	81.5
0.55	43.6	44.0	44.5	44.7	0.95	83.0	82.8	82.8	82.4
0.56	44.6	45.0	45.5	45.7	0.96	84.0	83.8	83.8	83.4
0.57	45.7	46.0	46.5	46.6	0.97	85.0	84.7	84.8	84.3
0.58	46.6	47.0	47.4	47.5	0.98	85.9	85.8	85.7	85.2
0.59	47.6	47.9	48.3	48.4	0.99	87.0	86.7	86.5	86.2
0.60	48.5	48.9	49.4	49.4	1.00	88.0	87.6	87.5	87.1

7. Paraffin oil dissolves carbon dioxide in greater amounts than does water, and its action in preventing loss of carbon dioxide from blood is due to prevention of diffusion from the surface of the water rather than to the formation of a layer impermeable to gas. Consequently too thick a layer of oil should not be used and the tube should be subjected to a minimum of agitation after the blood is in it.

8. The apparatus needs to be carefully mounted on the board, so that there is no excessive strain on the parts.

9. It is desirable to keep the amount of caprylic alcohol small (about 0.02 c.c.) as larger amounts may appreciably increase the results, because of the vapor tension of impurities which the alcohol may contain, and because it dissolves much more air per unit volume than does water.

10. Catch the water residue and mercury overflow in a flask. It requires only washing with water, drying with filter paper and straining through cloth or chamois skin to prepare the mercury for use again.

11. Practically the only source of difficulty with the determination is the entrance of air through the stopcocks. It is essential that both cocks should be properly greased and air tight (see below). It is also necessary that the cocks (especially the lower one) should be held in place by rubber bands so that they cannot be forced out by pressure of the mercury.

12. The 1 per cent ammonia solution is of some benefit in keeping the apparatus clean. For thorough cleaning remove the rubber tubing and fill the apparatus by suction with *aqua regia* and let stand several hours.

13. In the determination of the carbon dioxide content of plasma, the plasma is transferred from the centrifuge tube directly into the cup of the apparatus. The determination should be made as soon as possible after withdrawal of the blood.

14. If 1 c.c. of plasma is not available, 0.5 c.c. may be used, in which case the volume of distilled water and acid used is halved so that the total volume of water solution introduced is only 1.25 c.c., and in the calculation the *observed* volume of gas is multiplied by 2.

15. The determination can be performed on whole blood if, in place of sulphuric acid, lactic acid is used, made by diluting 1 volume of concentrated acid (specific gravity 1.20) to 10 volumes with water.

16. As the gas is being brought to atmospheric pressure, the meniscus of water over the mercury should be raised slowly in the narrow part of the apparatus so that there be no oscillation of the column and resulting excessive reabsorption of carbon dioxide. With faulty technic, 0.01 to 0.02 c.c. of carbon dioxide may be reabsorbed.

17. Van Slyke and Stadie give the following directions for introducing blood into the apparatus: The cup is rinsed out with distilled water before each determination and then 1 c.c. of distilled water is placed in the cup. The blood or plasma is run under this layer of water. A drop of caprylic alcohol is added and the blood or plasma followed by the layer of water is run into the

chamber of the apparatus, leaving only the drop of alcohol in the capillary above the cock; the 0.5 c.c. of acid is measured into the cup and run through into the chamber.

STOPCOCK LUBRICANT FOR VAN SLYKE APPARATUS.—One part of pure, unvulcanized, para rubber gum finely divided by scissors is dissolved by the aid of heat in 5 parts of vaselin. Keep in small ointment jars.

A thin layer of vaselin is first uniformly applied to the cock and the latter is fitted and turned several times. The rubber lubricant is then applied in the same manner. In warm weather relatively little vaselin is used; in cold weather more is needed. The two lubricants used in this manner have proved more satisfactory than a single lubricant made by dissolving the rubber gum with larger amounts of vaselin.

DETERMINATION OF CHLORIDES

Principle.—The principle of the Volhard method is employed, that is, precipitation of silver chloride with silver nitrate and titration of the excess silver nitrate by means of thiocyanate, using ferric ammonium sulphate as an indicator.

Reagents.—1. STANDARD SILVER NITRATE.—Weigh out on an analytical balance 2.905 grams C.P. silver nitrate; wash into a beaker using a stream of water from a wash bottle; when dissolved, transfer to a liter volumetric flask and dilute to the mark with distilled water. Preserve in a brown bottle. As organic material reduces silver nitrate to silver, contact with rubber or cork is undesirable. One c.c. equals 1 milligram of sodium chloride.

2. STANDARD THIOCYANATE.—Because thiocyanates are hygroscopic, the standard solution must be prepared volumetrically. Ammonium or potassium thiocyanate may be used. As an approximation, dissolve about 1.4 grams ammonium thiocyanate or 1.7 grams potassium thiocyanate in a liter of distilled water and by titration (as given below) and by proper dilution, adjust so that 5 c.c. are equivalent to 5 c.c. silver nitrate solution.

3. CONCENTRATED NITRIC ACID (specific gravity 1.42).

4. POWDERED FERRIC AMMONIUM SULPHATE.—The solid ferric ammonium sulphate is used rather than a solution in order to insure a high concentration in the mixture to be titrated. It is powdered in order to facilitate its solution.

Procedure (Whitehorn).—1. Pipet 10 c.c. of protein-free tungstic acid filtrate into a 50 c.c. porcelain casserole.

2. Add 5 c.c. of standard silver nitrate solution and mix thoroughly.

3. Add about 5 c.c. of concentrated nitric acid (graduated cylinder); mix and let stand for five minutes to permit the precipitation of silver chloride.

4. Add, with a spatula, about 0.3 gram of ferric ammonium sulphate.

5. Titrate (not too slowly) the excess silver nitrate with standard thiocyanate solution in a micro buret until the definite salmon red (not yellowish)

color of the ferric thiocyanate persists in spite of stirring for at least fifteen seconds.

6. CALCULATION:

$$x = \text{c.c. of ammonium thiocyanate or potassium thiocyanate used}$$

$$(5 - x)100 = \text{milligrams sodium chloride in 100 c.c. blood}$$

NOTES.—1. The normal concentration of the chlorides in whole blood ranges from 450 to 520 milligrams per 100 c.c. In the plasma, the concentration ranges from 570 to 620 milligrams per 100 c.c.

2. Chlorides of the blood are increased in some cases of nephritis, and in some cardiac conditions; while low values have been observed in fevers, pneumonia, severe diabetes, and after the administration of diuretics.

3. The determination of blood chlorides is of practical value as an indication or contra-indication for a salt-free diet.

4. All glassware used must have been washed with distilled water and all reagents must be halogen free. Some samples of nitric acid contain much chloride. Sodium tungstate may contain halogens and is tested as follows: Mix 1 volume 10 per cent sodium tungstate with 2 volumes concentrated chloride free nitric acid; filter into test tube containing a silver nitrate solution. Turbidity indicates halogens.

5. It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction.

6. The use of nitric acid is the essential point in the procedure, for aside from preventing the precipitation of silver phosphate, it flocks out the silver chlorides with a consequent reduction of the surface exposed. As silver thiocyanate is more insoluble than silver nitrate, it is evident that the surface of silver chloride exposed must be made as small as possible in order to prevent reaction between silver chloride and ferric thiocyanate. The abundance of ferric ammonium sulphate used also retards this reaction by reducing the ionization of the latter. This also deepens the end-point color by preventing the ionization of the red salt, $Fe(CNS)_3$, into yellow Fe and colorless CNS ions.

7. Mixture of tungstic acid and chloride brings down more silver than can be accounted for by chloride alone, but the possibility for error is avoided by carrying on the titration in the presence of the precipitate when all the silver which has not been precipitated by chloride is available for titration with thiocyanate.

8. Whitehorn's greatest deviations with the method were −1.3 and +1.2 per cent and the limit of error with his careful technic was therefore less than 1.5 per cent. He used volumetric flasks to make the 1 : 10 dilution. An error of 0.1 c.c. in measurement of silver nitrate will cause about 4 per cent error in

the final result and an error of 0.1 c.c. in measurement of thiocyanate will result in about 2 per cent error. Obviously the burets must be clean and not too large.

9. The method is applicable to plasma as well as whole blood, but loss of carbon dioxide must be prevented until plasma has been separated from corpuscles.

DETERMINATION OF INORGANIC PHOSPHORUS

Principle.—The blue color obtained by adding molybdic acid and a reducing agent to an inorganic phosphate solution is compared colorimetrically with a solution similarly prepared from a standard phosphate solution.

Reagents.—Ten times normal sulphuric acid (10 N · H_2SO_4) prepared as follows: 450 c.c. concentrated H_2SO_4 added to 1300 c.c. water.

MOLYBDATE NO. 1.—12 5 grams ammonium molybdate in 100 c.c. of water. To a 500-c.c. graduated cylinder add 250 c.c. 10 N · H_2SO_4 and the molybdate solution; dilute to the mark.

MOLYBDATE NO. 2.—Prepare as above adding only 150 c.c. of acid.

MOLYBDATE NO. 3.—Molybdate as above without any acid. Discard when considerable amount of sediment appears.

10 PER CENT TRICHLORACETIC ACID.—100 grams diluted to 1000 c.c. with distilled water.

15 PER CENT SODIUM BISULPHITE —75 grams diluted to 500 c.c. Let stand two to three days to free of turbidity. Filter. Keep well stoppered.

20 PER CENT SODIUM SULPHITE.—Dissolve 50 grams in 95 c.c. of water. Filter. Keep well stoppered.

0.25 PER CENT AMINONAPHTHOSULPHONIC ACID.—Dissolve 0.5 gram of dry powder in 195 c.c. of the sodium bisulphite solution. Add 5 c.c. of the sulphite solution. Stopper and shake until dissolved. If bisulphite is old, cloudiness results and more sulphite is required; add in 1 c.c. quantities, until clear, being careful not to add too much. This solution will keep at least two weeks.

STANDARD PHOSPHATE SOLUTION.—Dissolve 0.3509 gram of potassium dihydrogen phosphate in water and transfer quantitatively to a 1-liter volumetric flask. Add 10 c.c. of 10 N · H_2SO_4 and dilute to the mark. Mix. Will keep indefinitely. 5 c.c.=0.4 mg. P.

To PURIFY AMINONAPHTHOLSULPHONIC ACID.—Buy the technical product from Eastman Kodak Company. Heat 1000 c.c. water to 90° C. and dissolve in it 150 grams sodium bisulphite and 10 grams crystalline sodium sulphite. Add 15 grams technical sulphonic acid and shake until all but amorphous impurities have dissolved. Filter. Cool under running water. Add 10 c.c. concentrated hydrochloric acid; filter with suction; wash with 300 c.c. of water and finally with alcohol until washings are colorless. Air dry without exposure to light. Preserve in a brown bottle.

BLANK PHOSPHATE CORRECTION.—Arrange three 150 c.c. beakers on a white paper. To *A*, add 100 c.c. water. To *B*, 85 c.c. water; 10 c.c. of molybdate No. 1, and 4 c.c. of sulphonic reagent. To *C*, 40 c.c. of trichloracetic acid; 45 c.c. water; 10 c.c. molybdate No. 2, and 4 c.c. of sulphonic reagent. Stir. Into *B* add 1 c.c. quantities of a phosphate solution (.005 mgm. per c.c. made by diluting 5 c.c. of the standard phosphate solution to 80 c.c.) Stir after each addition and allow to stand two minutes until each have the same blue color. The number of c.c. of this phosphate multiplied by 0.05 is the correction to be subtracted from final result in analysis of blood. Solution *A* should be colorless.

Procedure (Fiske and Subbarow).—To a 50-c.c. Erlenmeyer flask add 4 volumes of trichloracetic acid and while gently rotating add 1 volume of blood. Close with a stopper, shake vigorously a few times and filter through an ashless filter. Measure 5 c.c. of the filtrate into a 10-c.c. volumetric flask or graduated cylinder, add 1 c.c. of molybdate No. 2 and mix. Add 0.4 c.c. of sulphonic reagent, dilute to 10 c.c. and mix. At the same time prepare the standard solution by transferring exactly 5 c.c. of the phosphate standard to a 100 c.c. volumetric flask. Add 10 c.c. of molybdate No. 1 and 4 c.c. of the sulphonic reagent. Mix after each reagent is added. Dilute to the mark. Make the reading in five minutes but if particularly strong repeat later.

In case of a low phosphorous under 2 milligrams per cent the determination may be saved by adding a known amount of phosphate from the standard before diluting the unknown to the mark and subtracting from the final result.

CALCULATION.—When the cup of the unknown is set at 10 millimeters, the reading of the standard cup multiplied by 0.4 gives milligrams of inorganic phosphorus per 100 c.c. of blood.

NOTES.—1. In adults the inorganic phosphorus is about 3 milligrams in 100 c.c. of plasma. Children have a higher content, about 5 milligrams in 100 c.c. There is an increase of this phosphorus in cases of nephritis with acidosis, and a slight increase during bone repair after major fractures. In active rickets, the phosphorus is regularly reduced. In tetany, the phosphorus may be normal or reduced.

2. This method may be applied to food, tissue, and other analyses. Dry ashing with nitrate and carbonate may be used when desired. Magnesium nitrate is sometimes good for urines, using 1 c.c. of a 10 per cent solution. This will not hurt platinum unless an appreciable amount of sodium and potassium is present. Silica does not interfere and thus ordinary crucibles may be used.

3. The blood should preferably be collected in sodium citrate, as oxalated bloods give values about 10 per cent too low (Buell).

4. Analysis should be started immediately after drawing of blood. Standing of clotted or defibrinated blood even in the ice box results in a diminution of inorganic and increase in organic phosphorus. Oxidation results in migration of phosphates from serum into corpuscles (Lawaczck).

5. The method is not applicable to whole blood or to serum or plasma of badly hemolyzed blood. This is due to the fact that the strong heating and acid will cause hemolysis of organic phosphorus compounds contained in the corpuscles.

6. Longer heating than ten minutes will produce some increase in color in both standard and filtrate, but after ten minutes the proportionality is not changed.

METHOD FOR DETERMINATION OF CALCIUM

Principle.—The calcium precipitated as oxalate is determined by titrating the oxalic acid, liberated by acid, with standard permanganate.

Reagents.—1. POTASSIUM PERMANGANATE (N/10 and N/100).—All glassware which comes in contact with potassium permanganate should be freed from organic matter by cleaning in a sulphuric acid dichromate mixture. See Chapter XXX for method of preparation.

2. N/10 (SODIUM OXALATE).—See Chapter XXX for preparation.

3. SULPHURIC ACID (approximately normal).—Add 28 c.c. concentrated sulphuric acid to a liter flask about half full of distilled water. Cool to room temperature and dilute to the mark with water.

4. AMMONIUM OXALATE C.P. (4 per cent solution).—It is best not to keep a large amount of stock solution since it readily undergoes hydrolysis, yielding ammonium hydroxide which attacks the glass, and because decomposition occurs into ammonium carbonate and carbon monoxide.

5. AMMONIA (2 per cent).—Two c.c. concentrated C.P. ammonia plus 98 c.c. distilled water. It is best not to make up more than 100 c.c. at a time.

Procedure (Clark and Collip).—To be carried out in duplicate:

1. Pipet into a *clean* 15 c.c. conical Pyrex centrifuge tube: 2 c.c. fresh serum, 2 c.c. distilled water, and 1 c.c. 4 per cent ammonium oxalate solution. Mix with a stirring rod.

2. Allow to stand thirty minutes or longer.

3. Centrifuge until the precipitate is well packed in the bottom of the tube.

4. Pour off the supernatant liquid carefully, and while the tube is still inverted, place it in a rack for five minutes to drain with the mouth of the tube resting on a piece of filter paper.

5. Wipe the mouth of the tube dry with a piece of filter paper.

6. With a fine stream from a wash bottle stir up the precipitate and wash the sides of the tube with 3 c.c. of dilute ammonia water.

7. Centrifuge and drain as before.

8. Add 2 c.c. normal sulphuric acid by blowing the acid out of the pipet directly upon the precipitate so as to break up the mat.

9. Place in a boiling water bath for about a minute.

10. Titrate the oxalic acid with N/100 potassium permanganate, using a microburet graduated in 0.02 c.c. and carrying out the titration over a white

background with the tube partly immersed in an 800 c.c. beaker containing water at a temperature of 70° to 75° C.

11. CALCULATION:

C.c. N/100 potassium permanganate × 10 = milligrams calcium per 100 c.c. serum

NOTES—1. The normal range is about 9 to 11 milligrams in 100 c.c. of serum. In children and infants it is slightly higher. In tetany and after parathyroidectomy, there is a decrease. Low calcium is frequently found in acute rickets, pneumonia, and in same cases of epilepsy.

2. The centrifuge tubes should be perfectly clean. They should be kept in potassium dichromate sulphuric acid cleaning solution.

3. As the tap water contains considerable calcium (about 4 milligrams per 100 c.c.) the bloods need to be collected in syringes and test tubes which have been rinsed with distilled water and dried. All glassware used in the determination must be rinsed with distilled water.

4. To insure uniform drainage, the centrifuge tubes are thoroughly cleaned each time before use by immersion in a cleaning solution of concentrated sulphuric acid and potassium dichromate.

5. In titrating, the permanganate should be added very slowly at the beginning, as it takes a little time for the reaction to start and oxygen will be lost if permanganic acid accumulates. The second drop should not be added until the pink color given by the first drop has disappeared. The titration temperature is important and should be 70° to 75° C. at the start and not lower than 60° C. at the end. Otherwise too much permanganate will be used. The centrifuge tube may be conveniently held in the water bath with a test-tube holder and may be stirred by giving it a gently whipping motion. The end-point is to be taken as the faintest persisting pink color that can be recognized when looking down the tube against a white background; at this point no pink color is recognized if one looks through the tube.

6. The subtraction of a permanganate blank is omitted. The method is arbitrary and the procedure must be closely adhered to. Clark and Collip say that with careful work errors can be kept under 2 per cent.

DETERMINATION OF CHOLESTEROL

Principle.—Blood absorbed on filter paper is extracted with chloroform to remove cholesterol and cholesterol esters. Cholesterol, including that of cholesterol esters, is determined colorimetrically by the Liebermann-Burchard reaction (acetylation by acetic anhydride and condensation by concentrated sulphuric acid).

Reagents.—1. C.P. CHLOROFORM.

2. ACETIC ANHYDRIDE (C.P.).—Keep in a glass stoppered container. "Merck's reagent" in one-quarter pound bottles is recommended. If necessary

the acetic anhydride may be redistilled, the distillate being collected between 134° C. and 140° C.

3. SULPHURIC ACID (C.P.; concentrated).

4. CHOLESTEROL STANDARD STOCK SOLUTION.—Use *dry* glassware. Weigh on an analytical balance 0.08 gram pure dry cholesterol and dissolve in 100 c.c. pure dry chloroform in volumetric flask. Preserve in a tightly stoppered amber bottle.

5. WORKING STANDARD (0.4 milligram per 5 c.c.).—Pipet 25 c.c. of stock standard into 250 c.c. volumetric flask and dilute with pure dry chloroform. Preserve in a tightly corked amber bottle. This solution keeps well.

Apparatus.—1. Special Leiboff extraction tubes, with side tube; graduated at 5 c.c. (Empire Laboratory Supply Company).

2. Filter paper disks, prepared from fat-free filter paper, having a diameter of three-quarter inch and thickness of one-sixteenth inch, are recommended. Ordinary filter paper extracted with chloroform gives a slight color with the reagents. Suitable disks can be prepared by extracting No. 5 Whatman filter paper with ether in a Soxhlet extractor. When dry, the paper is cut with scissors.

Procedure.—Lieboff's Method.—1. Put about 5 c.c. dry chloroform into a dry special Lieboff extraction tube.

2. Drop filter paper disk in place.

3. Pipet 0.25 oxalated whole blood on the filter paper disk. The blood is immediately absorbed by the paper and no drying is necessary.

4. With a cork stopper attach the extraction tube to a reflux condenser and heat on an electric hot plate or immerse in a beaker of boiling water. Extract for thirty minutes.

5. Detach tube from condenser, remove paper disk and cool by immersing in cold water for a minute. When cool, add chloroform exactly to the 5 c.c. mark.

6. In a similar tube place 5 c.c. of the cholesterol standard solution.

7. Add with pipet to both standard and unknown: 2 c.c. acetic anhydride, 0.1 c.c. concentrated sulphuric acid.

8. Insert clean cork stoppers in the tubes and invert twice to mix. Place the tubes in a dark place for ten minutes.

9. Compare in the colorimeter with the standard set at 20.

10. CALCULATION:

$$\frac{20}{R} \times 160 = \text{milligrams per 100 c.c. whole blood}$$

Method Using Plaster of Paris (Myers and Wardell).—1. Pipet 1 c.c. of blood into about 5 grams of plaster of Paris in a glass mortar. Mix thoroughly and place in drying oven at 105° C. for one hour.

2. Transfer the pulverized dried mass to the extraction thimble. Place from 20 to 25 c c. of chloroform in the extraction apparatus (Figs. 288 and 289) and extract for ninety minutes on the electric hot plate.

3. Transfer the chloroform extract to one of the 25 c.c. flasks, rinse and dilute to the mark with chloroform.   Mix.

4. Pipet 10 c.c. of the cholesterol standard into a dry test tube.   Into other dry test tubes pipet 10 c c. of the chloroform extracts.   To each add 2 c.c. of acetic anhydride and o 2 c c. of sulphuric acid.   Place the test tubes near the colorimeter in the light by which the reading is to be made and allow to stand for fifteen minutes. Compare in the colorimeter.

5. CALCULATIONS :

S = reading of standard

R = reading of blood extract

x = milligrams of cholesterol per 100 c c. of blood

$$x = \frac{200S}{R}$$
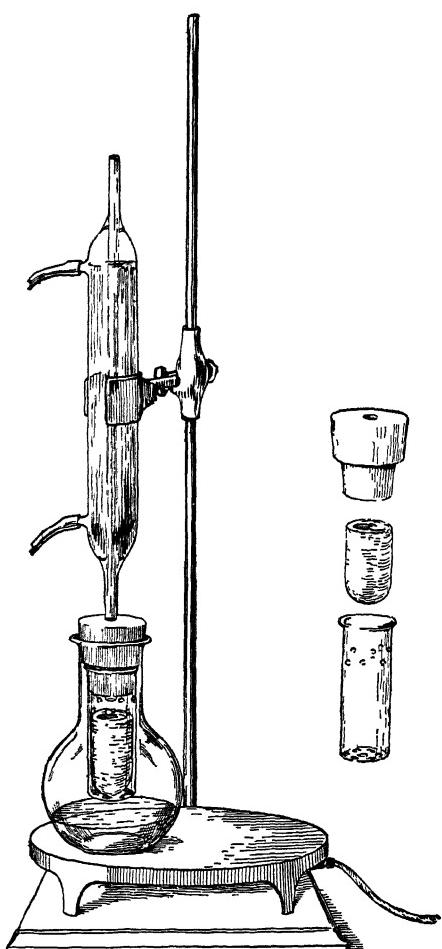
FIG. 288.—EXTRACTION APPARATUS FOR CHOLESTEROL DETERMINATION

(After Myer, *Practical Chemical Analysis of Blood,* C V. Mosby Co)
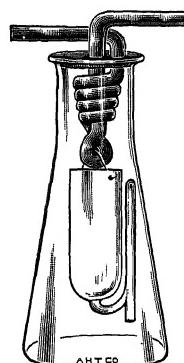
FIG. 289 —EXTRACTION APPARATUS FOR CHOLESTEROL DETERMINATION.

6. **Alternate Method.**—Set the cup of the unknown at 10 millimeters when the reading of the standard multiplied by 20 equals milligrams of cholesterol per 100 c c. of blood.

NOTES —1. After reaching its greatest intensity the cholesterol color fades rather rapidly. It is important that the reagents be anhydrous and that all

glassware used be dry. Poor acetic anhydride in particular results in weak color development.

2. The normal concentration of cholesterol in the blood ranges from 140 to 180 milligrams per 100 c.c. of blood.

3. In order that the final color may be free from turbidity, all reagents must be anhydrous (chloroform and acetic anhydride redistilled). All glassware must be absolutely dry.

4. Cholesterol is partly endogenous and partly exogenous; the latter particularly from eggs, butter, meats, and some vegetables.

5. Cholesterol has been found increased in diabetes with lipemia, nephritis, complete obstruction of the common bile duct, during pregnancy and in some cases of cholethiasis and arteriosclerosis. Decreased concentration has been observed in pernicious anemia, cachexia of malignancy and in some cases of high fever.

A COLORIMETRIC METHOD FOR THE DETERMINATION OF PLASMA PROTEINS

Principles.—1. There are two steps in the determination of the plasma proteins: (*a*) their separation from each other, and (*b*) their quantitative estimation.

2. Solutions of pure serum globulin or albumin may, of course, be used for the standard. But as these are laborious to prepare and difficult to keep, a solution of tyrosine is employed. The color reaction of proteins with the reagent originally devised for estimation of phenols is employed, and is due to the presence of tyrosine in the proteins. A convenient standard is made by dissolving 50 milligrams of tyrosine in 250 c.c. of N/10 hydrochloric acid.

3. Under the conditions described below it has been found for human plasma that 1 milligram of tyrosine equals 11.3 milligrams of fibrin (N×6.25); 15.8 milligrams of globulin, or 21.4 milligrams of albumin.

4. The tyrosine equivalents of the plasma proteins of different species of animals are quite different.

5. In the method which follows, the fibrin and the albumin are determined directly, while the globulin is determined by the difference between the total serum proteins and the albumin. All the determinations can be made simultaneously.

Determination of Fibrin (Wu and Ling).—Reagents.—1. *Sodium Chloride Solution* (0 8 per cent).

2. *Calcium Chloride Solution* (2.5 per cent).

3. *Sodium Hydroxide Solutions* (1 per cent and 10 per cent).

Keep these three well stoppered and discard if a precipitate develops.

4. *Sulphuric Acid Solution* (5 per cent).

5. *Standard Tyrosine Solution*—Dissolve 50 milligrams of tyrosine (Pfanstiehl) in 250 c.c. of N/10 hydrochloric acid. Use a volumetric flask. Wu states: "We have observed no change in this solution in the course of 6 months and it may keep much longer."

6. *Phenol Reagents.*—Dissolve 100 grams of sodium tungstate (Na₂WO₄ · 2H₂O), and 25 grams of sodium molybdate (Na₂MoO₄ · 2H₂O), in 700 c.c. of water. Add 50 c.c. of 85 per cent phosphoric acid, and 100 c.c. of concentrated hydrochloric acid. Boil with reflex condenser for eight hours, and dilute to 1 liter.

Procedure.—1. Pipet 1 c.c. of plasma from oxalated blood (containing 0.2 to 0 6 per cent potassium oxalate) into a 50 c.c. beaker.

2. Add 28 c.c. of 0.8 per cent sodium chloride solution.

3. Then add 1 c.c. of 2.5 per cent calcium chloride solution.

4. Mix and allow to stand undisturbed for twenty minutes or as long as necessary to cause the jelly to stick to a glass rod.

5. Transfer the jelly to a slender glass rod with pointed end by gently whirling the rod. All the fibrin can be made to stick to the rod. If difficulty is experienced, dry the fibrin on the rod with filter paper and then catch the other masses of jelly on the end of the rod.

6. Partially dry the fibrin on the rod with filter paper and then slip the fibrin off and press it between dry filter paper to remove the water as completely as possible.

7. Transfer the small mass of fibrin to a 15 c.c. centrifuge tube and add 4 c.c. of 1 per cent sodium hydroxide solution.

8. Place the tube in a boiling water bath and stir with a very slender glass rod until the lump has completely disintegrated. Calcium oxalate remains in suspension.

9. Add 10 c.c. of water; mix and centrifuge.

10. Pour off the supernatant liquid into a 25 c.c. glass-stoppered graduated cylinder.

11. Cool under the tap and add 1 c.c. of 5 per cent sulphuric acid.

12. Pipet 1 c.c. of the standard tyrosine solution into a 25 c.c. glass-stoppered cylinder. The standard and the unknown are now treated alike as follows:

13. Add 0.5 c.c. of phenol reagent.

14. Dilute to about 20 c.c.

15. Add 1 c.c. of 10 per cent sodium hydroxide solution.

16. Mix by gentle rotation, make up to volume, and mix.

17. Let stand for one hour before making the color comparison.

18. Calculation when the standard is set at 20:

$$\frac{20}{R} \times 0.226 = \text{per cent of fibrin per 100 c.c. blood plasma}$$

NOTES.—1. If the amount of fibrin in the plasma is very high (0.8 per cent or more), the fibrin jelly will not shrink rapidly. In such case it is necessary to use less plasma for the determination.

2. If the per cent volume of cells with reference to whole blood is known, the values may be readily calculated for whole blood.

Determination of Albumin.—Procedure.—1. To 1 c.c. of plasma add 4 c.c. of distilled water and 5 c.c. of saturated ammonium sulphate solution, or 9 c.c. of saturated magnesium sulphate solution and 0.3 gram of anhydrous magnesium sulphate.

2. Mix and allow to stand for thirty minutes. Filter.

3. Measure 1 c.c. of the filtrate into a 15 c.c. centrifuge tube. Add about 12 c.c. of water, 1 c.c. of 10 per cent sodium tungstate solution, and then 1 c.c. of two-thirds normal sulphuric acid. It is advisable to add one or two more drops of acid.

4. Stir thoroughly with a slender glass rod and centrifuge.

5. Carefully decant the supernatant liquid as completely as possible.

6. The volume of the wet precipitate usually amounts to about 0.5 c.c. If it is much smaller than 0.5 c.c., indicating low albumin, measure another c.c. of albumin filtrate into the same tube, dilute with water, and proceed as before. Add to the precipitate in the centrifuge tube 1 c.c. of sodium tungstate solution. Stir until the precipitate has dissolved, dilute with 13 c.c. of water, and add 1 c.c. of two-thirds normal sulphuric acid.

7. Stir again, centrifuge, and decant the supernatant liquid. This second precipitation is intended to remove the calcium and ammonium or magnesium so nearly completely that they cannot possibly interfere with the subsequent color reaction, although experience has shown that a single precipitation usually suffices.

8. Add to the precipitate in the tube 10 c.c. of water and 1 or 2 drops, but not more, of 20 per cent sodium carbonate solution. Stir until the precipitate has dissolved.

9. Transfer the resulting solution to a 25 c.c. volumetric flask or graduated tube.

10. Rinse the centrifuge tube twice with 3 c.c. of water. Add 0.5 c.c. of phenol reagent and 1 c.c. of 10 per cent sodium hydroxide solution. Mix by gentle rotation. Make to volume and mix. Prepare a standard as in the fibrin determination and read the color after one hour.

Determination of Albumin and Globulin.—Procedure.—1. Measure 2 c.c. of the filtrate in the fibrin determination into a 15 c.c. centrifuge tube and proceed exactly as in the determination of albumin.

2. CALCULATION: In the calculation it is to be noted that the solution used for the albumin determination is plasma diluted 1:10, while that used for the determination of albumin and globulin is plasma diluted 1:30. If 1 c.c. of the albumin solution is used for the former determination and 2 c.c. of the serum solution are used for the latter determination, and the colorimeter readings are R_a and R_t respectively, the standard being set at 20, then the total apparent tyrosine in 1 c.c. of serum is:

$$15 \times \frac{20}{R_t} \times 0.2 \text{ milligram};$$

the apparent tyrosine of albumin in 1 c.c. of serum is:

$$10 \times \frac{20}{R_a} \times 0.2 \text{ milligram;}$$

and the apparent globulin in 1 c.c. of serum is:

$$\left(15 \times \frac{20}{R_t} \times 0.2\right) - \left(10 \times \frac{20}{R_a} \times 0.2\right) = \frac{60}{R_t} - \frac{40}{R_a} \text{ milligrams}$$

Since 1 milligram of tyrosine equals 15.8 milligrams of globulin or 21.4 milligrams of albumin of human serum:

$$\text{Per cent of globulin} = \frac{\dfrac{60}{R_t} - \dfrac{40}{R_a} \times 15.8}{1000} \times 100 = \left(\frac{6}{R_t} - \frac{4}{R_a}\right) \times 15.8$$

$$\text{Per cent of albumin} = \frac{\left(10 \times \dfrac{20}{R_a} \times 0.2\right) \times 21.4}{1000} \times 100 = \frac{20}{R_a} \times 4.28$$

DETERMINATION OF CAROTIN

Principle.—Carotin-colored serum has an orange tint while bilirubin gives it more of a straw color.

Procedure (Johnson).—1. The clear serum, practically free from corpuscles, which presses out when freshly clotted blood is allowed to clot without being disturbed, is completely desiccated with an excess of plaster of Paris.

2. The powdery mass is then moistened to a thick paste with absolute or 98 per cent alcohol and thoroughly shaken with low boiling-point petroleum ether (30° to 50° C.).

3. Yellow discoloration of the petroleum ether signifies carotin. (Extracting pigment from alcohol-moistened serum by means of petroleum ether is equivalent to extracting the pigment from 80 to 90 per cent alcohol by petroleum ether and indicates its carotin nature. But little carotin can be extracted from serum by direct shaking with fat solvents.)

4. The following test may also be used but is not as delicate as the above: Lipochromes, including carotin, are precipitated with proteins when 2 volumes of 95 per cent alcohol are added to 1 volume of serum while bilirubin remains in the supernatant fluid when the precipitated proteins are centrifugalized.

DETERMINATION OF OXYGEN CAPACITY

1. The Van Slyke blood gas apparatus (see Fig. 287) is washed out twice with water before each analysis in order to remove the alkali used to absorb carbon dioxide in any previous analysis.

2. The entire apparatus is filled with mercury, including the capillaries above the upper stopcock. For 2 c.c. of blood, introduce 6 c.c. of water, 0.3 c.c. of 1 per cent saponin (Merck) solution and 2 or 3 drops of caprylic alcohol into the apparatus, and free of air by evacuation and fifteen seconds of shaking. The extracted air is expelled and the extraction is repeated until no more air is obtained.

3. The air-free solution is now drawn down and trapped in the wide branch of the apparatus below the lower stopcock. The stopcock is turned and mercury run very slowly upwards through the apparatus in order to collect the film of water left on the inside and this film is expelled through the outlet capillary on the left side. Mercury is now run into the bottom of the cup and any moisture in the cup is dried by filter paper.

4. Two c.c. of oxalated blood are now drawn into the 50-c.c. chamber from a pipet by lowering the mercury reservoir, and are trapped near the bottom of the chamber. While the upper stopcock remains open, the apparatus is shaken for two to three minutes, thus saturating the blood with oxygen.

5. The mercury is run up again into the 50-c.c. chamber, collecting the blood at the top. When the blood reaches the upper stopcock, this is closed. The lower stopcock is turned so that the previously trapped air-free water is allowed to rise in the chamber. The lower stopcock is closed and the apparatus shaken a few seconds to mix the water and blood. The blood is laked in one and one-half minutes.

6. From 0.10 to 0.12 c.c. of potassium ferricyanide solution (20 grams per 100 c.c. water) is measured into the cup and introduced into the chamber with the laked blood. (The ferricyanide may be measured with sufficient accuracy as 3 drops from a dropper which delivers 1 c.c. in 25 to 30 drops.) A mercury seal is made and the apparatus is evacuated and shaken for two to three minutes.

7. The evolved gas is composed of oxygen, nitrogen and carbon dioxide. In order to absorb the carbon dioxide the leveling bulb is placed at such a height that the mercury in it is slightly below the level of the mercury in the apparatus, and 0.5 c.c. of 0.5 N sodium hydroxide solution (previously saturated with air by shaking) is admitted from the cup of the apparatus and allowed to trickle *slowly* down the inner wall of the chamber. If the latter part of the solution enters as a solid column instead of running down the walls, it is dislodged by letting a little mercury pass down through it in a fine stream. In any case it is usually necessary to dislodge with a drop of mercury the last drop of alkali solution which adheres just below the stopcock.

8. The fluid mixture is trapped in the bulb below the lower stopcock, mercury is run up through the left arm and the reading is made in the same manner as in the determination of carbon dioxide.

9. CALCULATION: The volume of gas measured (V) is $O_2 + N_2 + H_2O$ and correction must be made for temperature (t) barometric pressure (B), water

vapor (W) and physically dissolved air (2.1 c.c. of air being dissolved in 100 c.c. of blood at 20° C.).

$$V\left(50\times\frac{B-W}{760(1+0.00367t)}\right)-2.1 = \text{volume per cent oxygen capacity}$$

The value in parentheses is a factor which can be obtained from the table.

TABLE FOR THE CALCULATION OF HEMOGLOBIN CONTENT

Volumes per cent oxygen capacity $\times$ 0.746 = grams hemoglobin per 100 c c. blood.   Volumes per cent oxygen capacity $\times$ 4 78 = per cent hemoglobin *

Temperature, Centigrade	Factor $= 50\times B-W/760(1+0.00367t)$
15	46 6 $\times B/760$
16	46 4 $\times B/760$
17	46.2 $\times B/760$
18	45 95 $\times B/760$
19	45.75 $\times B/760$
20	45 5 $\times B/760$
21	45.3 $\times B/760$
22	45.05 $\times B/760$
23	44 85 $\times B/760$
24	44.6 $\times B/760$
25	44.4 $\times B/760$
26	44.15 $\times B/760$
27	43 9 $\times B/760$
28	43 65 $\times B/760$
29	43 4 $\times B/760$
30	43 15 $\times B/760$

* Based on Haden's average normal of 15 6 grams

Blood can be kept in an ice box at 60° C. for at least twenty-four hours before any appreciable change takes place. After a certain time blood will absorb oxygen through the oil, and the values will increase. The opposite happens when the blood is kept in the laboratory where it cannot be expected to keep constant more than two hours. After that interval the oxygen content diminished rapidly, probably on account of bacterial action. (Lundsgaard, *J. Biol. Chem.*, 1918, 33 : 143.)

Oxygen Unsaturation.—1. Volume per cent oxygen unsaturation equals volume per cent oxygen capacity minus volumes per cent oxygen bound by hemoglobin in venous blood.

2. This latter determination is made in the same manner as the oxygen content but the calculation is changed as follows:

$$V\left(50\times\frac{B-W}{760(1+0.0)0367t}\right)-1.5 = \text{volumes per cent oxygen}$$

bound by hemoglobin in venous blood.

DETERMINATION OF METHEMOGLOBIN

Principle.—The method depends upon the fact that both hemoglobin and methemoglobin are changed quantitatively to cyanhemoglobin by dilute solutions of potassium cyanide. The color of the latter is a brilliant orange-red and is very suitable for colorimetric comparison. The change of methemoglobin to cyanhemoglobin is rapid even in the cold. Hemoglobin, however, changes slowly at room temperature, and this difficulty is avoided by converting all the hemoglobin present into methemoglobin by use of potassium ferricyanide and then converting the methemoglobin into cyanhemoglobin. The resulting solution of cyanhemoglobin is compared with a standard of known strength in a Duboscq colorimeter.

The total amount of hemoglobin plus methemoglobin having thus been determined colorimetrically, the hemoglobin content of the blood containing the two pigments (hemoglobin and methemoglobin) is determined separately from the oxygen capacity, employing the gasometric technic of Van Slyke. The hemoglobin determined by the oxygen capacity is subtracted from the hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is methemoglobin.

Standard.—1. The hemoglobin content (grams per 100 c.c.) is determined gasometrically.

2. Then place 10 c.c. of oxalated or defibrinated blood, which is known to contain no methemoglobin, in a 500 c.c. flask.

3. Hemolyze by adding 300 c.c. water.

4. Add 2.5 c.c. of 3 per cent potassium ferricyanide solution and let stand twenty minutes.

5. Now add 25 c.c. of a 0.1 per cent potassium cyanide solution and dilute to 500 c.c.

The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

Example of calculation: Strength of standard equals 15 grams hemoglobin per 100 c.c. blood. Comparison of cyanhemoglobin in colorimeter: Standard 10, unknown 12. Unknown has 10/12 of 15 or 12.5 grams of total blood pigment per 100 c.c. Gasometric determination of hemoglobin equals 10 grams per 100 c.c. Therefore, sample has 12.5 minus 10 or 2.5 grams of methemoglobin per 100 c.c.

Procedure.—1. Two c.c. of oxalated whole blood are placed in a 100 c.c. flask and 50 c.c. of water are added, which produces hemolysis in a few seconds.

2. Add 0.5 c.c. of a M/10 (3 per cent) solution of potassium ferricyanide, and let stand twenty minutes.

3. Now add 5 c.c. of a 0.1 per cent potassium cyanide solution. The change to cyanhemoglobin is immediate.

4. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin which is expressed as grams of "total hemoglobin pigment" per 100 c.c. of blood.

5. Determine the oxygen capacity by the gasometric method of Van Slyke and multiply by 0.746 to obtain grams of hemoglobin per 100 c.c.

6. CALCULATION: Total hemoglobin pigment per 100 c c. minus hemoglobin per 100 c.c. which can bind oxygen equals methemoglobin per 100 c.c.

DETERMINATION OF IRON

Principle.—The organic matter is destroyed with perchloric-sulphuric acid mixture, the iron in the resulting solution treated with sulphocyanate and the color compared with a standard iron solution.

Reagents.—1. CRYSTALLINE FERROUS AMMONIUM SULPHATE.

2. AMYL ALCOHOL.

3. CONCENTRATED SULPHURIC ACID.

4. PERCHLORIC ACID (60 per cent; bought in this concentration).

5. CONCENTRATED NITRIC ACID.

6. POTASSIUM SULPHOCYANATE (20 per cent).

Standard Solution.—Exactly 3.5 grams of ferrous ammonium sulphate are dissolved in about 100 c.c. of water in a liter volumetric flask. Forty c.c. of 10 per cent sulphuric acid are added, the solution warmed, and approximately N/10 potassium permanganate added dropwise until a faint, permanent pink color is obtained. Cool and dilute to the mark. One c.c. of this solution is equal to 0.5 milligram of iron.

Procedure (Kennedy's Modification of Wong's Method).—1. One c.c. of blood in a 100 c.c. Kjeldahl flask is digested with 5 c.c. of concentrated sulphuric acid and 2 c.c. of 60 per cent perchloric acid over a micro-burner in the same manner as in the nonprotein nitrogen determination. After about ten minutes the solution should be colorless and sulphur trioxide fumes should be evolved. Cool; add 1 drop of concentrated nitric acid; transfer quantitatively to a 100 c.c. volumetric flask and dilute to the mark.

2. One c.c. of standard solution is treated as above.

3. Ten c.c. of each solution are then pipetted into 50 c.c. ground-glass stoppered mixing cylinders and to each are added 10 c.c. of amyl alcohol and 5 c.c. of 20 per cent potassium sulphocyanate. Shake until the color is completely extracted by the amyl alcohol and compare in the colorimeter.

4. CALCULATION: If the standard is set at 20 millimeters:

$$\frac{1000}{R} = \text{milligrams iron in 100 c.c. blood}$$

NOTES.—1. The tubes used in the nonprotein nitrogen digestion may be used instead of Kjeldahl flasks.

2. It is important that the digestion of the standard be carried out exactly as the blood. In this way allowance is made for the iron in the reagents.

3. Amyl alcohol enhances the strength and quality of the final color and makes possible more accurate colorimeter comparisons.

4. The standard recommended is for normal blood. Hemoglobin below 80 is best compared against a proportionately weaker standard made by properly diluting the standard given.

CHAPTER XXXII

METHODS FOR CHEMICAL EXAMINATION OF URINE

COLLECTION AND PRESERVATION OF URINE

Only special methods are given in this chapter as the ordinary routine chemical methods for urinalysis are given in Chapter VI.

1. If any dependable data are desired regarding the quantitative composition of the urine, the examination of the mixed excretion for twenty-four hours is generally necessary. In collecting the urine the bladder may be emptied at any given hour, *e.g*, 8 A M., the urine discarded and all the urine voided from that hour up to and including that passed the next day at 8 A.M. saved, thoroughly mixed and a sample taken for analysis.

2. Powdered thymol is not a wholly satisfactory preservative, evidence having been presented to show that it interferes with the quantitative estimation of sugar, acetone, and diacetic acid. It also may give a confusing reaction in the nitric acid test for albumin.

3. Toluene overlayed on the urine is a very satisfactory preservative.

4. In certain pathological conditions, it is desirable to collect both day and night specimens. Urine voided between 8 A M. and 8 P.M. may be taken as the day sample and that voided between 8 P.M. and 8 A.M. as the night sample.

5. The qualitative testing of urine samples collected at random, except in a few specific instances, is of no particular value in so far as giving accurate knowledge of the exact urinary characteristics. In the great majority of cases, a sample of the mixed twenty-four-hour specimen will give the most helpful data.

DETERMINATION OF SUGAR

Principle.—Urine is heated with an alkaline dinitrosalicylic solution and the resulting color is compared with a standard equivalent to a standard glucose solution.

Reagents.—1. DINITROSALICYLIC ACID.—Dissolve 10 grams in water and dilute to 1 liter (1 per cent).

Place 300 c.c. of 4.5 per cent sodium hydroxide in a 2 liter graduated cylinder; add 880 c.c. of 1 per cent dinitrosalicylic acid and 255 grams of sodium potassium tartrate. Mix until dissolved. Preserve in a brown bottle in the dark.

2. PERMANENT STANDARD SERIES—Dissolve 0.8625 gram of ferric ammonium sulphate in water and dilute to 100 c.c. in a volumetric flask. Into

each of twelve 100 c.c. volumetric flasks add 10 c.c. of the 1 per cent dinitro-salicylic acid solution and add in order 1, 1.75, 2.53, 3.22, 4, 4.68, 5.32, 6.10, 6.80, 7.5, 9.1, 10.3 c.c. Dilute each to 100 c.c. and mix. Transfer portions of each to tubes similar to those used in doing the test and label these tubes respectively, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.5, 3 per cent. These tubes will then read directly in percentage of sugar considering the 1 : 10 dilution as made in the test.

Procedure (Sumner).—1. Into a small test tube, pipet 1 c.c. of the urine to be examined; add 9 c.c. of water with a pipet or by diluting to a 10 c.c. mark on the tube. Mix.

2. Pipet 1 c.c. of this diluted urine into a Folin sugar tube or to a test tube graduated with a line at 25 c.c.

3. Add 3 c.c. of the reagent and place in boiling water for five minutes. Cool in running water, dilute to mark, and mix.

4. Compare with the standard tubes and read directly the percentage from the tube. In case it is stronger than 3 per cent, dilute with a definite proportion of water, generally equal parts, and multiply the answer by the dilution factor.

NOTES.—1. Slightly increased accuracy may be obtained by using an artificial or glucose standard and the colorimeter.

2. In the above method the qualitative test may be combined with the quantitative. All urines reading 0.2 per cent or under may be considered *negative* for glucose.

3. Sugar in normal urine may be read by using undiluted urine in the above test when the percentage of sugar will be that given on the standard tubes divided by 10.

4. Urines showing 0.25 per cent or over will give a positive Benedict qualitative test.

Scopometer Modification of Sumner's Test.—1. Mix 0.2 c.c. of urine with 10 c.c. of reagent in a test tube.

2. Heat five minutes in boiling water.

3. Allow to cool.

4. Place test tube in scopometer (see Chapter XXIX) and examine with green filter; read result from calibration.

5. If the urine contains a large amount of sugar, dilute the test five or ten times with water and multiply by the number of dilutions.

6. This modification has the following advantages: (*a*) The manipulations take no more time and trouble than the usual qualitative tests. (*b*) It is exceedingly sensitive and accurate. (*c*) It covers the whole range from none to 15 per cent or more sugar without the necessity of repeating the test. (*d*) It is critical in the zone between normal and pathological sugar, *i e.*, 0.2 to 0.3 per cent.

Reagent.—1. Dissolve 200 grams of sodium potassium tartrate (Rochelle salts) in about 1000 c.c. of warm distilled water.

2. Dissolve 24 grams of sodium hydroxide in about 200 c.c. of distilled water.

3. Add 2 to 1 and let cool.

4. Add to this mixture 6 grams of phenol and 6 grams of sodium bisulphite.

5. Dissolve 14 grams of 3.5 dinitrosalicylic acid (Eastman) in about 200 c.c. of boiling water. When dissolved, let cool somewhat and add to the previous solution and make up to 2000 c.c. by distilled water.

DETERMINATION OF UREA AND UREA NITROGEN

Principle.—See blood urea (p. 528).

Procedure (Van Slyke and Cullen's Modification of Marshall's Method).
—1. Measure the specimen and note whether it is a twenty-four-hour specimen or a specimen for urea tolerance test. Make a determination of free ammonia (see below) at the same time this determination is run.
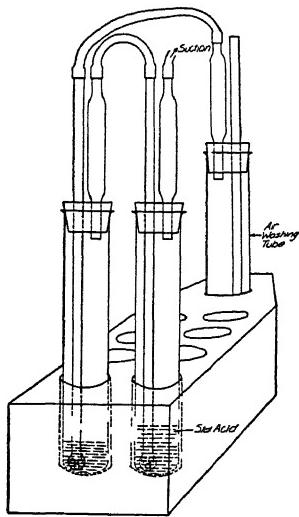
FIG 290 —AERATION APPARATUS.

2. Pipet 5 c.c. urine into a 100 c.c. volumetric flask and dilute to mark.

3. With a pipet transfer 5 c.c. of the diluted urine to a thick-walled, large Pyrex test tube (200 by 25 millimeters).

4. Add two powdered urease tablets (25 milligrams each, Hynson, Westcott and Dunning).

5. Stopper and let stand at room temperature for one-half hour.

6. Add 2 drops of caprylic alcohol.

7. Add 5 c.c. of saturated potassium carbonate solution.

8. Draw off the ammonia by suction aeration (Fig. 290) into another tube containing 20 c.c. N/100 hydrochloric acid and 2 drops of methyl red indicator. Aeration should last about one hour and during the first two minutes only a slow current of air should be used.

9. Titrate the residue of acid with N/100 sodium hydroxide.

10. CALCULATION: Each c.c. of acid neutralized indicates 0.00014 gram of nitrogen.

$$\left(20 - \text{c.c.}\ \frac{N}{100}\ \text{sodium hydroxide}\right) \times 4 \times 0\ 00014 \times \text{volume of urine in c.c.}$$

$$= \text{grams urea nitrogen} + \text{ammonia nitrogen}$$

or

$$\left(20 - \text{c.c.}\ \frac{N}{100}\ \text{sodium hydroxide}\right) \times 0.00056 \times \text{volume of urine in c.c.}$$

$$= \text{grams urea nitrogen} + \text{ammonia nitrogen}$$

(Grams urea nitrogen + ammonia nitrogen) − grams ammonia nitrogen

$$= \text{grams urea nitrogen}$$

Grams urea nitrogen × 2 145 = grams urea

DETERMINATION OF AMMONIA NITROGEN

Procedure.—1. Pipet 5 c.c. of urine into a large Pyrex test tube.

2. When ready for aeration add 5 c.c. saturated solution of potassium carbonate and drive off ammonia into 20 c.c. N/100 hydrochloric acid.

3. Titrate the excess acid with N/100 sodium hydroxide.

4. Calculation:

$$\left(20 - \text{c c.} \frac{N}{100} \text{ sodium hydroxide}\right) \times \frac{0\ 00014}{5} \times \text{volume of urine in c c.}$$
$$= \text{grams ammonia nitrogen}$$

or

$$\left(20 - \text{c.c.} \frac{N}{100} \text{ sodium hydroxide}\right) \times 0.000028 \times \text{volume of urine in c c.}$$
$$= \text{grams ammonia nitrogen}$$

DETERMINATION OF UREA

Principle.—Urine is treated with permutit to remove ammonia, filtered, and the filtrate is used for the urea determination by means of Karr's urea nitrogen method.

Reagents.—PERMUTIT.

UREA NITROGEN (STOCK).—Dissolve 0.1286 gram of urea in water and dilute to 200 c.c. (5 c.c. contain 1.5 milligrams urea nitrogen.)

UREA NITROGEN STANDARD FOR USE.—Place 5 c.c. of the stock urea solution in a 100-c.c. volumetric flask and dilute to mark. (5 c.c. contain 0.075 milligrams urea nitrogen.)

PHOSPHATE BUFFER.—Dissolve 14 grams of the sodium pyrophosphate and 2 grams of the metaphosphoric acid in water and dilute to 250 c.c.

UREASE.—Place 15 grams of Jack bean meal and about 5 grams of permutit in a large Erlenmeyer flask and add a mixture of 16 c.c. of alcohol and 84 c.c. of water. Shake gently but continuously for ten to fifteen minutes. Allow to stand overnight and filter into small flasks. Keep in refrigerator, tightly stoppered.

MERCURIC POTASSIUM IODIDE.—In a large Erlenmeyer flask (about 500 c.c.) place 75 grams of potassium iodide, 55 grams of iodine, 50 c.c. of water and 75 grams of mercury. Shake continuously until the iodine color begins to fade. Cool under running water with continued shaking until the color of the iodine has changed to the greenish color of the double iodide. Decant into a liter graduate. Wash the residue several times with water, decanting each washing into the graduate. Dilute the solution and washings to 1 liter. Filter if the solution is not perfectly clear.

NESSLER.—Place 350 c.c. of 10 per cent sodium hydroxide in a 500-c.c. flask, add 75 c.c. of the mercuric potassium iodide solution, and dilute with water to the mark. Place in a reservoir bottle the stopper of which has a tube containing soda lime to protect the Nessler solution from carbon dioxide. Use from tube at lower outlet, discarding the solution contained in the tip on first use in the day.

Procedure (Keller).—To 15 or 25 c.c. of urine in an Erlenmeyer flask, add 5 grams of permutit. Shake. Allow to stand ten minutes. Filter. Pipet 1 c.c. of filtrate into a liter volumetric flask and dilute to the mark with distilled water. Pipet 5 c.c. into a plain test tube; add 5 drops of urease and 5 drops of buffer.

Pipet 5 c.c. of urea nitrogen standard into a plain test tube; add 5 drops of urease and 5 drops of buffer.

Digest both test tubes in water bath at 50° C. for ten minutes. Transfer contents of each tube to specially marked graduated tubes, washing over with water and dilute to 22.5 c.c. Add Nessler's to 25 c.c. mark. Compare in colorimeter.

CALCULATION.—Set unknown at 16 millimeters. Reading of standard multiplied by 0.2 is per cent urea or grams per 100 c.c. of urine. Total urea in grams is per cent multiplied by liters multiplied by 10.

DETERMINATION OF TOTAL NITROGEN

Principle.—The urine is diluted and the determination is then carried out as is the nonprotein nitrogen determination of blood.

Procedure (Haden's Modification of Folin and Denis Method).—1. Measure the volume of the twenty-four-hour specimen; take the specific gravity and test for albumin.

2. Pipet 5 c.c. urine into a 100 c.c. volumetric flask. (The pipet is to be drained fifteen seconds against the wall of the flask and then blown.)

3. Dilute to the mark with distilled water and mix.

4. With a pipet, transfer 1 c.c. of the diluted urine to a dry, 200 by 25 millimeters, thin-walled, lipped Pyrex test tube graduated at 35 c.c. and 50 c.c.

5. Add a dry glass bead and 1 c.c. digestion mixture.

6. Proceed as with blood.

7. CALCULATION:

$$\frac{20}{R} \times 0.3 = \text{grams total nitrogen per 100 c.c. urine}$$

$$\text{Grams total nitrogen per 100 c.c.} \times \frac{\text{c.c. volume of 24-hour specimen}}{100}$$

$$= \text{grams total urinary nitrogen per twenty-four hours}$$

NOTE.—If the urine is concentrated with a specific gravity above 1.030, use 2 c.c. urine, in which case calculation is made by the following formula:

$$\frac{20}{R} \times 0.75 = \text{grams total nitrogen per 100 c.c. urine}$$

Method for Checking Accuracy of Colorimetric Method.—Principle.— Macro-Kjeldahl method using a digestion mixture containing sulphuric acid, phosphoric acid and copper sulphate.

Reagents.—1. *Acid Digestion Mixture.*

Sulphuric acid (C.P. special, low nitrogen)................2126 c.c. (43 per cent)
Phosphoric acid (C.P., 85 per cent)......................1624 c.c. (32 per cent)
Cupric sulphate (C.P., 3 per cent) ($CuSO_45H_2O = 4.7$ per cent 1250 cc. (25 per cent)

2. *Sodium Hydroxide Solution.*—Saturated solution = 55 grams per 100 c.c. solution. (Use sodium hydroxide, purified flake, for nitrogen determination.)

3. *N/10 Hydrochloric Acid and N/10 Sodium Hydroxide.*

Procedure.—1. Pipet 5 c.c. of urine in a 500 c.c. Kjeldahl flask.

2. Add 20 c.c. of digestion mixture.

3. Digest over low flame until colorless on a micro-burner.

4. Turn on full flame until precipitate forms and digestion is complete (usually requires about fifteen minutes).

5. Cool and dilute to about 250 c.c. with distilled water.

6. Add a little talc powder (purified) to prevent bumping.

7. As receiving bottle use a 500 c.c. Erlenmeyer flask to which has been added 40 c.c. N/10 hydrochloric acid and 2 or 3 drops of methyl red indicator.

8. Make the solution in the Kjeldahl flask alkaline to phenolphthalein with about 50 c.c. saturated sodium hydroxide solution.

9. Immediately connect the Kjeldahl trap and condenser and boil until 180 to 200 c.c. have distilled over (exercise care that distillate does not suck back).

10. When nearly finished, lift the tip of the condenser from the receiving bottle and allow the distillate to drop into the bottle for the last five minutes in order to wash out the condenser. (Distillation usually requires about thirty minutes.)

11. Wash the tip of the receiving tube with distilled water.

12. Titrate excess acid with N/10 sodium hydroxide.

13. CALCULATION:

40 — c.c. N/10 sodium hydroxide × 0.028 = grams nitrogen per 100 c.c.

MICROMETHOD FOR DETERMINING NITROGEN

Principle.—Digestion is conducted with a special acid mixture of high oxidizing power followed by direct nesslerization, thus avoiding the usual processes of distillation and aeration.

Procedure (Rose).—1. A special digestion tube is used, made from a Pyrex test tube, 25 by 250 millimeters, whose bottom is drawn to an obtuse point like that of the usual 15 c.c. centrifuge tubes. This tapering end is bent at an angle of about 20 degrees to the long axis of the tube. The acid mixture consists of 100 c.c. of concentrated sulphuric acid and 160 c.c. of 60 per cent perchloric acid made up to 500 c.c. with ammonia-free water.

2. Measure a sample judged to contain from 0.25 to 2.0 milligrams of nitrogen, into the special Pyrex tube. Add 1 c c. of acid mixture and a solid glass bead to minimize bumping. Hold the tube over the free flame of a micro-burner in such a way as to expose a maximum of the liquid's surface. Boil until the mixture becomes dark-colored and heavy fumes evolve. Add 3 drops of hydrogen peroxide solution. Insert a small funnel into the mouth of the digestion tube and turn it so as to expose a minimum of surface of the liquid Heat in such a manner that the liquid will simmer; when digestion is nearly complete, as shown by the disappearance of nearly all of the brownish color, add 3 drops of hydrogen peroxide,[1] to destroy the last trace of color. Heat from three to five minutes after it is clear. Add 3 or 4 c c. of water, add 8 c.c. of 2 N hydroxide, dilute to 50 c.c., and mix. Add 5 c.c. of Nessler's reagent[2] diluted to 25 c.c. and mix. Dilute to the 80- or 100-c.c. mark. Match against a nesslerized solution of 0.5, 1, or 1.5 milligram of ammonium sulphate per c.c.

3 The time required for digestion varies with the nature of the sample, from two minutes after clear for a diluted urine, to more than eight minutes for blood protein coagulum, or a fatty semisolid sample. Boiling off water requires one minute per c c. The time of heating after clear depends on the nature of the substances present and can best be left to the judgment of the individual analyst who will be guided in this by his experience with macro-Kjeldahl operations or by a few preliminary tests. To obtain a blank on the reagent, 1 c.c. of the 1 milligram standard ammonium sulphate solution and a few grains of cane sugar are digested as if this were a sample of urine. The analyst may encounter samples of too low a nitrogen content to warrant dilution, and rich in substances which make a viscid, frothy mess difficult to

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[1] The hydrogen peroxide is "perhydrol" diluted 1·5 In the simplest cases, such as urinary nitrogen determinations, the hydrogen peroxide may be omitted The hydrogen peroxide hastens the oxidation, and it helps in managing frothy digestions

[2] The following quantities are used in making up 1 liter of Nessler's solution: Mercuric iodide, 100 grams; potassium iodide, 70 grams; sodium hydroxide, 100 grams. Many formulas for making up Nessler's solution are to be found in the literature Inasmuch as the degree of alkalinity affects the color value, the alkali should be kept within narrow limits (about 2 c c. of N/10 by adhering to some one formula). This also applies to the neutralization of the digested sample.

digest. It is then better to give up the advantage of direct nesslerization. Double or treble the amount of acid mixture, use hydrogen peroxide more liberally, dilute the digested sample to 10 or 12 c.c., insert a stopper with aeration tubes into the mouth of the digestion tube, connect with a sulphuric acid scrubber and a receiving cylinder containing 2 c.c. of N/10 sulphuric acid, aerate and heat the digestion tube with a micro-burner. By this combination of aeration and distillation the ammonia will be transferred to the receiving cylinder in about three minutes. It is then nesslerized in the usual way.

4. This procedure will give all the nitrogen expected from regular Kjeldahl methods. It will not give all of the guanidine and creatinine nitrogen.

DETERMINATION OF CHLORIDES

Principle.—The principle of the Volhard method is employed: precipitation of silver chloride with a known amount of silver nitrate and titration of the excess silver nitrate by means of thiocyanate using ferric ammonium sulphate as an indicator.

Reagents.—1. SILVER REAGENT.—Dissolve 29.042 grams silver nitrate in 150 c.c. water. Dissolve 150 grams ferric alum in 350 c.c. water with the aid of heat. Cool somewhat and transfer both solutions to a liter volumetric flask. Add 400 c.c. concentrated nitric acid. Cool to room temperature and dilute to mark. One c.c. equals 10 milligrams sodium chloride.

2. AMMONIUM THIOCYANATE SOLUTION —Dissolve about 14 grams ammonium thiocyanate in a liter of water. Mix thoroughly. Place in the buret. Place 10 c.c. of the silver reagent in a 100 c.c. flask, add 20 c.c. water and titrate to the first permanent salmon color.

$$t = \text{c.c. thiocyanate titration}$$

To a 1000 c.c. volumetric flask add $1000 - 100t$ c.c. of water and add the thiocyanate solution to the mark. Mix. The solution should be exactly equal to the silver. Check by repeating the titration.

Procedure (Volhard-Harvey).—Transfer 5 c c. of urine to a 100 c.c. flask; add 20 c.c. water, and 10 c.c. of silver reagent. Titrate with thiocyanate from the buret. The first permanent salmon-red color is the end-point.

CALCULATION:

$$t = \text{c.c. thiocyanate titration}$$

$$(10 - t) \times 0.01 \times \frac{\text{volume of urine in c c.}}{5} = \text{grams of sodium chloride}$$

or

$$(10 - t) \times 0\ 002 \times \text{volume of urine in c.c.} = \text{grams of sodium chloride}$$

NOTES.—1. The sodium chloride in the urine depends on the amount eaten in the diet. It is useful in checking the efficacy of a salt-free diet.

2 The rate of chloride excretion is useful as a kidney functional test.

QUANTITATIVE ESTIMATION OF BILE ACIDS

Principle.—This method is based upon the principles of the method proposed by Szilárd for the estimation of bile acids in the blood, and the fact that bile acids are precipitated from solution by saturating with magnesium sulphate.

Reagents.—1. FERRIC CHLORIDE.—One per cent of ferric chloride containing 0.05 per cent of concentrated hydrochloric acid.

2. SULPHOSALICYLIC ACID.—Eight grams of sulphosalicylic acid in 1000 c.c. of N/10 hydrochloric acid.

Procedure (Katayama).—An alcoholic solution of the bile acids of the urine, prepared by adding 50 c.c. of absolute alcohol to 10 c c. of urine, is heated in a boiling water bath to effect solution of the bile acids and to coagulate the proteins. This is filtered and the filtrate is evaporated at about 70° C. to dryness *in vacuo* The residue is dissolved in about 15 c.c. distilled water and the bile acids are precipitated from their aqueous solution by the addition of solid magnesium sulphate to saturation. After standing in the ice box overnight, the residue is filtered on the best quality filter paper, and then washed with small quantities of a cold saturated solution of magnesium sulphate. After the filter paper has been dried completely, the bile acids are extracted overnight with 80 c.c. of absolute alcohol. This filtrate is evaporated *in vacuo* to 10 c.c., and 200 c.c. of absolute ether are added to the residue. This mixture is allowed to stand overnight. The bile acids are precipitated by this alcohol-ether mixture. The supernatant fluid is removed by siphon without disturbing the precipitate. When entirely dry, the precipitate is dissolved in 5 c.c. of water, transferred to a 15 c.c. centrifuge tube, and 0.3 c.c. of 1 per cent ferric chloride solution is added and the solution thoroughly mixed. On standing in the incubator at 38° C. overnight, the bile acids combine with the ferric chloride and are precipitated as ferric salts. The mixture is centrifuged and the supernatant fluid removed by siphonage. After the precipitate has been washed twice with water, it is dissolved in 5 c.c. of absolute alcohol and quantitatively transferred to a test tube, using 3 c.c. absolute alcohol. To this test tube 5 c.c. of chloroform and 5 c c. of sulphosalicylic acid reagent are added. After thoroughly mixing, the stoppered tube is permitted to stand in a bath at 40° to 50° C. with occasional shaking for two to three hours, *i.e.*, until the purple color in the upper layer has reached its maximum intensity.

A stock solution of 0.1 per cent glycocholeic acid may be kept for about one week. From this stock solution a series of four standards for comparison are prepared by pipetting into 15 c c. conical centrifuge tubes 2, 1 5, 1, and 0.5 c.c., respectively, of the stock solution. The contents of each tube is diluted to 5 c.c. and to this solution 0.3 c.c. of the ferric chloride reagent is added. The stoppered tubes containing the standard solutions are incubated at about 40° C. overnight. From this point the procedure for the development of the color in the standards is identical with that for the unknown. Have available

four standards for comparison in the colorimeter, containing 2, 1.5, 1, and 0.5 milligram of glycocholic acid.

To obtain the most satisfactory results it has been found advisable to keep the colorimetric reading within a narrow range, about 15 millimeters. This is accomplished by a comparison of the unknown with the standard most closely approximating it in color intensity. A sample from the upper colored layer of both standard and unknown is pipetted into the colorimeter cups for comparison.

CALCULATION:

S = milligrams of bile acid in the standard used for comparison

$$\frac{15}{R} \times S \times \frac{\text{volume of urine}}{\text{used urine volume}} = \text{milligrams of bile acids as glycocholeic acid per volume of urine}$$

CHAPTER XXXIII

METHOD FOR THE DETERMINATION OF BASAL METABOLIC RATE

Principle.—The diminution in volume of oxygen in a spirometer, in closed circuit with an individual's lungs and with soda lime to absorb the carbon dioxide, is measured and reduced to normal temperature and pressure. From this volume is calculated the twenty-four-hour calorie production and its positive or negative percental variation from the twenty-four-hour heat production of the normal individual having the same biometric measurements.

Materials.—APPARATUS.—Closed circuit respiration apparatus, with mouthpiece (sterilized by boiling), nose clip, thermometer, kymograph with recording paper and ink. The Benedict-Roth-Collins or Sanborn-Benedict basal metabolic apparatus are recommended.

Bed, couch, stretcher, or other object upon which patient may recline comfortably. Watch. Barometer (observatory).

CHEMICALS.—Soda lime. Oxygen, pure, in compression cylinder.

Procedure.—The patient is given a light supper the night before the determination; is given no breakfast; is kept in bed; is taken to the metabolism station on a stretcher and is given absolute rest for a half hour before the determination.

Wrap recording paper tightly around kymograph drum and keep in place by means of paper stickers. Fill recording pen with ink.

By means of rubber tube, connect oxygen compression cylinder to the petcock on the apparatus for introducing oxygen gas. Admit oxygen into apparatus until pen is within a few centimeters of the edge of the paper drum. Shut lever on oxygen tank and petcock on apparatus. Connect patient with mouthpiece of apparatus and start motor. Adjust nose clip. Start kymograph revolving. Record temperature of gas in apparatus and pulse of patient. At the end of eight minutes remove nose clip and mouthpiece from patient. Stop motor and kymograph. Record temperature of gas in apparatus. Record barometric pressure. Determine patient's nude weight. Record sex of patient. Record age in years to nearest birthday.

CALCULATION.—*Using Benedict-Roth-Collins Apparatus.*—1. Remove recording paper from kymograph drum and draw a line through the lower ends of the respiration markings to the lower edge of the paper.

2. Read on the graph paper, graduated in millimeters, the amount of oxygen in the apparatus where the line crosses a minute graduation and the amount in the apparatus six minutes later. The difference between these two readings

is the amount of oxygen in millimeters consumed by the patient in six minutes. Multiply by 20.73 to give c.c. of oxygen consumed in six minutes.

3. Dividing this amount by 6 gives the amount consumed in one minute. Multiply by 60 to obtain hour volume. Multiply by 24 to obtain twenty-four-hour volume. Correct the volume of gas to the volume at standard conditions of temperature and pressure (760 millimeters and 273° absolute temperature).

4. Divide by 1000 to change c.c. to liters. Multiply by 4.825, the value in calories of 1 liter of oxygen at normal temperature and pressure. Determine the positive or negative percental variation of this number from the expected number of calories per twenty-four hours, *i e.*, multiply by 100, divide by the expected number of calories and from it subtract 100.

H = expected calories per day (Dreyer)[1]

W = nude weight in grams

A = age in years

F = factor for correcting gas volume to standard pressure and temperature[2]

$$H \text{ for men} = \frac{\sqrt[2]{W}}{0.1015 \times A^{0.1333}}$$

$$H \text{ for women} = \frac{\sqrt[2]{W}}{0.1127 \times A^{0.1333}}$$

Equation:

$$\frac{\text{millimeters} \times 20.73 \times F \times 60 \times 24 \times 4.825 \times 100}{6 \times 1000 \times H} - 100 = \text{basal metabolic rate}$$

Condensed equation: $\dfrac{\text{millimeters} \times 2400 \times F}{H} - 100 = \text{basal metabolic rate}$

Using the Sanborn-Benedict Apparatus.—Remove recording paper from kymograph drum. Draw a line from the left edge of the paper to the right through the upper ends of the respiration markings. Read on the graph paper, graduated in c.c., the amount of oxygen in the apparatus where the line crosses a minute graduation and the amount in the apparatus six minutes later. The difference between these two readings is the amount of oxygen consumed by the patient in six minutes. Dividing this amount by 6 gives the amount consumed in one minute. Multiply by 60 to obtain hour volume. Multiply by 24 to obtain twenty-four-hour volume. Correct the volume of gas to the volume at standard conditions of temperature and pressure. Divide by 1000 to change

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[1] See "Notes on Basal Metabolism. V. Tables of Values of Dreyer's Formulas," by W. H. Stoner; *Boston M. S. J.*, 1923, Vol. 189, No. 7, pp. 239–251.

[2] See T. M. Carpenter's *Tables, Factors and Formulas for Computing Respiratory Exchange and Biological Transformations of Energy*, 2nd ed., published by Carnegie Institution of Washington, D. C., 1924.

c.c. to liters. Multiply by 4.825, the value in calories of 1 liter of oxygen at normal temperature and pressure. Determine the positive or negative percental variation of this number from the expected number of calories per twenty-four hours, *i e*, multiply by 100; divide by the expected number of calories and from it subtract 100.

Equation ·

$$\frac{\text{c.c (of O}_2 \text{ per minute)} \times F \times 100 \times 60 \times 24 \times 4.825}{1000 \times H} - 100 = \text{basal metabolic rate}$$

Condensed equation: $\dfrac{\text{c c} \times F \times 695}{H} - 100 = \text{basal metabolic rate}$

Notes.—1. These directions are for the Benedict-Roth-Collins and the Sanborn-Benedict apparatus; the latter is equipped with a motor.

2. The Dreyer standards are recommended. The standards of Harris and Benedict or of Aub and DuBois may be used with slight modification of the above formulas.

3. A basal metabolic rate between −10 and +15 cannot be considered definitely pathologic.

4. The determination of basal metabolic rate is of clinical value in diagnosing and following the effect of treatment (surgical, radiologic, or medicinal) in hyperthyroidism and hyperpituitarism, in which conditions the rate is above normal; also in hypothyroidism and hypopituitarism, in which conditions the rate is below normal.

CHAPTER XXXIV

METHODS FOR THE CHEMICAL EXAMINATION OF MILK AND
OTHER FOODS

The methods herewith given for the chemical analysis of *cow's* milk are
taken from the fifth edition of *Standard Methods of Milk Analysis* of the
American Public Health Association and the Association of Official Agricul-
tural Chemists. Only those ordinarily requested of a clinical laboratory are
included. Some of these, with or without modifications, are employed for
the chemical analysis of *human* milk.

COLLECTION OF COW'S MILK

1. The quantity of sample required will depend upon the number of deter-
minations to be made. For the usual analysis collect 250 to 500 c.c. (½ to 1
pint) of sample; for the fat determination only, 50 to 60 c.c. (approximately
2 fluidounces) will suffice.

2. In the case of bottled milk, collect one or more bottles as prepared for
sale. In sampling bulk milk, thoroughly mix by pouring from one clean vessel
into another three or four times. If this procedure is impracticable, thor-
oughly stir the milk for at least one-half minute with a suitable appliance long
enough to reach to the bottom of the container. If cream has formed on the
milk, continue the mixing until all cream is detached from the sides of the
vessel and evenly emulsified throughout the liquid.

3. Place the samples in nonabsorbent, air-tight containers and keep them
in the cold, but at a temperature above freezing, until ready for examination.
When transported by mail, express, or otherwise, the containers should be
completely filled, tightly stoppered, and marked for identification. A neces-
sary quantity of preservative (corrosive sublimate, potassium dichromate, or
formaldehyde) may be used, except where the presence of the preservative
may be objectionable in connection with physical or chemical tests to be applied
in addition to the determination of fat.

4. Before withdrawing portions for analytical determinations, bring the
sample to a temperature of 15° to 20° C. and mix thoroughly by pouring into
a clean receptacle and back until a homogeneous mixture is assured. If lumps
of cream do not completely disappear, warm the sample to about 38° C., mix
thoroughly, then cool to 15° to 20° C. In case a measured volume is required
in a determination, bring the temperature of the sample to 20° C. before
pipetting.

BABCOCK METHOD FOR ESTIMATING THE FAT OF COW'S MILK

Principle.—Strong sulphuric acid is added to the milk to dissolve the serum solids and set free the fat from its emulsion The fat is then permitted to rise into the graduated neck of a Babcock bottle and the percentage read directly.

Reagent and Apparatus.—1. Commercial concentrated sulphuric acid with a specific gravity of 1.82 to 1.83 at 20° C.

2. Special Babcock milk pipet (Fig. 291) graduated to deliver 17.5 c c. but which holds 17.6 c.c. to the graduation mark, the extra 0.1 c.c. being allowed for the milk which clings to the walls.

3. Standard Babcock test bottle (Fig. 292).

4. Acid measure (Fig. 293) graduated to hold 17.5 c.c.

5. Centrifuge for the Babcock bottles. Special trunnion cups may be purchased for use with the International Centrifuge.

Procedure.—1. Transfer 17.6 c.c. (equivalent to 18 grams) of well-mixed milk to a milk test bottle by means of the special pipet. The milk remaining in the pipet tip shall be blown out.

2. Add 17.5 c c. of sulphuric acid, preferably not all at one time, pouring it down the side of the neck of the bottle in such a way as to wash any traces of the milk into the bulb. The temperature of the acid should be about 15° to 20° C.

3. Shake until all traces of curd have disappeared; then transfer the bottle to the centrifuge; counterbalance it; and, after the proper speed has been attained, whirl five minutes.

4. Add soft water at 60° C., or above, until the bulb of the bottle is filled.

5. Whirl two minutes.

6. Add hot water until the liquid column approaches the top graduation of the scale.

7. Whirl one minute longer at a temperature of 55° to 60° C. Transfer the bottle to the warm water bath maintained at a temperature of 55° to 60° C., immerse it to the level of the top of the fat column, and leave it there until the column is in equilibrium and the lower fat surface has assumed a final form.

8. Remove the bottle from the bath; wipe it; and measure the fat column, in terms of percentage by weight, from its lower surface to the highest point of the upper meniscus.

9. The fat column, at the time of measurement, should be translucent, of a golden yellow or amber color, and free from visible suspended particles. Reject all tests in which the fat column is milky or shows the presence of curd or of charred matter, or in which the reading is indistinct or uncertain.

ESTIMATION OF FAT IN CREAM.—1. Because of variation in specific gravity of cream and its high viscosity, 18-gram samples can be accurately taken only by weighing, but with cream containing between 20 and 30 per cent fat the sample is sufficiently accurate for routine work by measuring 18 c.c. of cream

with a special pipet. Use the special cream test bottle shown in Figure 294.

2. Proceed as with milk except that readings are made at the bottom of the upper meniscus. Liquid petrolatum added will flatten out the meniscus and make the reading easier.

NOTES.—1. In adding the acid, the test bottle is conveniently held at an angle so that the acid will run down the wall of the bottle and not in a small stream into the center of the milk, the bottle being slowly turned around and the neck thus cleared of adhering milk. The milk and the acid in the test bottle should be in two distinct layers without much of a black band of partially mixed liquids between them. Such a dark layer is often followed by

FIG 291 —SPECIAL BABCOCK MILK PIPET.

FIG. 292. — BABCOCK MILK TEST BOTTLE

FIG 293. — ACID MEASURE FOR THE BABCOCK METHOD
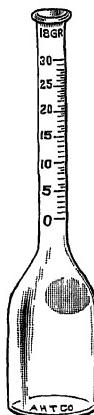
FIG. 294 — CREAM TEST BOTTLE.

an indistinct separation of the fat in the final reading. The cause of this may be that a partial mixture of acid and milk before the acid is diluted with the water of the milk will bring about too strong an action of the acid in this small portion of the milk, and thus char the fat contained therein. The appearance of black flocculent matter in or below the column of fat renders a correct measurement difficult and at times even impossible; if the black specks occur in the fat column itself, the readings are apt to be too high; if below it, the difficulty comes in deciding where the column of fat begins.

2. The acid should be carefully mixed with the milk by giving the test bottle a rotary motion. In doing this care should be taken that the liquid is

not shaken into the neck of the test bottle. When once begun the mixing should be continued until completed; a partial and interrupted mixing of the liquids will often cause more or less black material to separate with the fat when the test is finished. Clots of curd which separate at first by the action of the acid on the milk must be entirely dissolved by continued and careful shaking.

ESTIMATION OF TOTAL NITROGEN OF COW'S MILK

Principle.—Organic compounds are oxidized and the nitrogen converted into ammonia which is distilled off into a standard acid solution and titrated with standard alkali solution.

Procedure.—1. Place about 5 grams of the milk in a 500 c.c. Kjeldahl digestion flask.

2. Add approximately 0.7 gram of mercuric oxide, or 0.1 gram of crystallized copper sulphate, 10 grams of potassium sulphate (or 10 grams of anhydrous sodium sulphate) and 25 to 30 c.c. of sulphuric acid, specific gravity 1 84.

3. Place the flask in an inclined position and heat below the boiling point of the acid until frothing has ceased. (A small piece of paraffin may be added to prevent extreme frothing.) Then increase the heat until the acid boils briskly and digest for a time after the mixture is colorless or nearly so, or until oxidation is complete (Fig. 295).

4. Cool, dilute with about 200 c.c. of water, cool again, add a few pieces of granulated zinc or pumice stone, if necessary to prevent bumping, and 25 c.c. of a 4 per cent solution of potassium sulphide with shaking. (When no mercury or mercuric oxide is used the addition of the potassium sulphide solution is unnecessary.)

5. Next add sufficient saturated solution of sodium hydroxide free from nitrate, to make the reaction strongly alkaline (50 c c. are usually enough), pouring it down the side of the flask so that it does not mix at once with the acid solution.

6. Connect the flask by means of a Kjeldahl connecting bulb with a condenser (Fig. 296), mix the contents of the flask by shaking, and distill until all ammonia has passed over into a measured quantity of standard N/10 hydrochloric or sulphuric acid. The first 150 c.c. of the distillate will generally contain all the ammonia.

7. Titrate with N/10 standard alkali solution, using methyl red or cochineal solution as indicator.

8. Multiply the percentage of nitrogen by 6.38 to obtain the equivalent percentage to be reported as milk proteins.

9. Calculation:

$$\frac{50 - c\,c.\ N/10\ \text{sodium hydroxide} - c.c.\ \text{blank} \times 0.14}{\text{weight of sample}} = \text{per cent total nitrogen}$$

NOTES.—1. Determinations are to be made in duplicate and blanks are to be run, using about 1 gram of cane sugar instead of the unknown. Sugar aids in the reduction of any nitrates that may be present in the reagents.

2. The flame of the burner should strike only the portion of the flask below the level of the acid. Sheet iron or asbestos board with a hole in it serves well as a support.

3. The Kjeldahl flask should be fitted with a rubber stopper through which passes the lower end of a Kjeldahl connecting bulb to prevent sodium hydroxide being carried over mechanically during the distillation. The bulb should be about 3 centimeters in diameter and the tubes should be of the same diam-
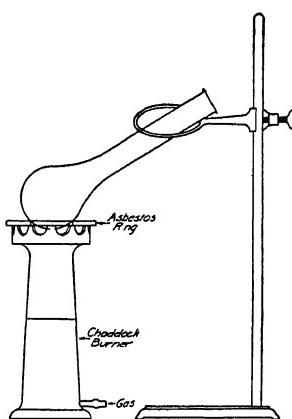
FIG 295—SUPPORT FOR KJELDAHL DIGESTION
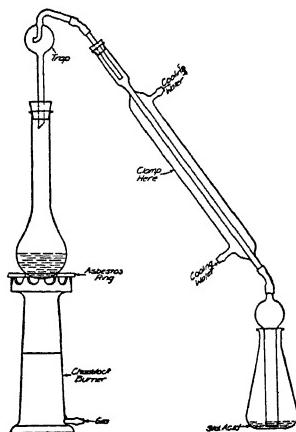
FIG 296 — APPARATUS FOR THE DISTILLATION OF AMMONIA FOR NITROGEN DETERMINATION.

eter as the condenser tube with which the upper end of the bulb is connected by means of rubber tubing. A piece of glass tubing about 12 centimeters long of the same diameter as the condenser tubing is attached to the lower end of the condenser by means of rubber tubing and should reach nearly to the bottom of the Erlenmeyer flask. This delivery tube is capable of being detached from the condenser for purposes of rinsing. It is preferable that the distilling system be made of Pyrex glass.

DETERMINATION OF CASEIN OF COW'S MILK

1. This determination should be made while the milk is fresh, or nearly so. When it is not practicable to make this determination within twenty-four hours, add 1 part of formaldehyde to 2500 parts of milk and keep in a cool place.

2. Place 10 grams of the sample in a beaker with 90 c.c. of water at 40°

to 42° C. and add at once 1.5 c c. of dilute acetic acid (1+9). Stir, and let stand three to five minutes.

3. Decant on a filter, wash by decantation two or three times with cold water, and transfer the precipitate to the filter. Wash once or twice on the filter.

4. The filtrate should be clear, or very nearly so. If the first portions of the filtrate are not clear, repeat the filtration, after which complete the washing of the precipitate.

5. Determine nitrogen in the washed precipitate and filter paper as described above for total nitrogen, and multiply by 6 38 to obtain the equivalent of casein.

6. To a sample of milk that has been preserved, the acetic acid should be added in small portions, a few drops at a time, with stirring, and the addition should be continued until the liquid above the precipitate becomes clear, or very nearly so.

DETECTION OF PRESERVATIVES IN COW'S MILK

Phenylhydrazin Test for Formaldehyde.—1. To a portion of the sample add an equal volume of strong alcohol, shake and filter from any insoluble matter.

2. To 5 c.c. of the filtrate add 0.03 gram of phenylhydrazin hydrochloride and 4 or 5 drops of a 1 per cent ferric chloride solution.

3. Mix, add slowly with agitation, in a bath of cold water to prevent heating the liquid, 1 to 2 c.c. of concentrated sulphuric acid.

4. Dissolve the precipitate by the addition of either concentrated sulphuric acid (keeping the mixture cool) or alcohol. In presence of formaldehyde a red color develops.

Ferric Chloride Test for Salicylic Acid.—1. Acidify 100 c.c. of the milk with 5 c.c. of hydrochloric acid (1+3), shake until curdled, filter, and extract with 50 to 100 c.c. of ether.

2. Wash the ether layer with two 5 c.c. portions of water, evaporate the greater portion of the ether in a porcelain dish on a steam bath, allow the remainder to evaporate spontaneously, and add a drop of 0.5 per cent ferric chloride solution.

3. A violet color indicates salicylic acid.

Test for Benzoic Acid.—1. Acidify, filter, and extract a 100 c.c. portion of the milk with ether as directed for salicylic acid. If benzoic acid is present in considerable quantity, it will crystallize from the ether in shining leaflets having a characteristic odor on heating.

2. Dissolve the residue in hot water, divide into two portions, and test as directed below. The residue may also be purified by sublimation and the melting point determined.

(a) Make the solution alkaline with ammonium hydroxide, expel the ex-

cess of ammonia by evaporation, dissolve the residue in water, and add a few drops of a neutral 0.5 per cent ferric chloride solution. A brownish precipitate of ferric benzoate indicates the presence of benzoic acid.

(*b*) Add to the water portion 1 or 2 drops of a 10 per cent solution of sodium hydroxide and evaporate to dryness. To the residue add 5 to 10 drops of concentrated sulphuric acid and a small crystal of potassium nitrate. Heat for ten minutes in a glycerol bath at 120° to 130° C., or for twenty minutes in a boiling water bath. The temperature must not exceed 130° C. After cooling add 1 c c. of water and make distinctly ammoniacal; boil the solution to decompose any ammonium nitrite that may have been formed. Cool and add a drop of fresh, colorless ammonium sulphide, without allowing the layers to mix. A red-brown ring indicates benzoic acid. On mixing, the color diffuses through the whole liquid and, on heating, finally changes to greenish-yellow. This differentiates benzoic acid from salicylic acid or cinnamic acid. The last two form colored compounds, which are not destroyed by heating.

Test for Borax and Boric Acid.—1. Immerse a strip of turmeric paper in the sample acidified with hydrochloric acid in the proportion of 7 c.c. of strong acid to each 100 c.c. of sample and allow the paper to dry spontaneously.

2. If borax or boric acid is present, the paper will acquire a characteristic red color, changed by ammonium hydroxide to a dark blue-green, but restored by acid.

COLLECTION AND CHEMICAL ANALYSIS OF HUMAN MILK

Collection.—There are two methods of obtaining samples of breast milk for analysis:

1. Express all the milk from one breast and mix thoroughly.

2. Draw 1 ounce of milk before nursing and 1 ounce after nursing. Mix the two samples thoroughly. The best time for obtaining samples is 9 to 10 A.M.

Determination of Specific Gravity.—Determined most conveniently by means of a Soxhlet, Veith or Quevenne lactometer at 60° F. The lactometer reading is corrected by adding 0.0001 for every degree F. above 60° and subtracting 0.0001 for every degree F. below this temperature.

Determination of Percentage of Fat.—This is essentially a modification of the Babcock test previously described except that a smaller bottle is employed (Fig. 297); the technic is practically the same. Otherwise the regular Babcock bottle and method are to be preferred if sufficient sample is available.

1. By means of a special narrow pipet, introduce milk up to the 5 c.c. mark.

2. Add sufficient commercial sulphuric acid (specific gravity 1.83) to fill the body of the tube and rotate to secure a homogeneous mixture.

3. Fill the neck of the tube with a mixture consisting of equal volumes

of amyl alcohol and concentrated hydrochloric acid and centrifuge for two minutes, etc, as described above.

4. The fat collects in a column in the upper part of the acid alcohol mixture and the percentage is read off directly.

Determination of Total Solids.—1. Introduce 2 to 5 grams of milk into a weighed flat-bottomed platinum dish and quickly ascertain the weight to milligrams.

2. Carefully expel most of the water by heating in a water bath and dry to constant weight in an oven regulated to a temperature between 100° and 105° C.

3. Divide the weight of the residue in grams by the weight of the milk used. Multiply the result by 100 to give the per cent of total solids in the milk.

Determination of Ash.—1. Heat the dry solids obtained above over a low flame (care should be taken that the dish is not heated above dull redness) until a white or light gray ash is obtained.

2. Cool the dish in a desiccator and weigh.

3. Divide the weight of the ash by the weight of the original milk taken for total solids and multiply by 100 to obtain the per cent of ash in the milk.

Determination of Total Nitrogen (Proteins).—1. Introduce 5 grams of milk into a 500 c.c. Kjeldahl flask and carry out the nitrogen determination as described above.

FIG. 297.—BABCOCK FAT BOTTLE FOR HUMAN MILK

2. Multiply the per cent of nitrogen by the factor 6.37 to obtain the protein content of the milk.

Determination of Lactose.—1. Introduce 1 c.c. of milk into a 100 c.c. volumetric flask.

2. Add 2 c.c. of 10 per cent sodium tungstate.

3. Add gradually 2 c.c. of two-thirds normal sulphuric acid or 16 c.c. of N/12 acid, mix well and let stand five minutes.

4. Dilute to the mark, mix and filter.

5. Into a Folin-Wu sugar tube introduce 1 c.c. of the filtrate and add 1 c.c. of water. Into another tube place 2 c.c. of standard lactose solution. Add 2 c.c. of the Folin-Wu alkaline copper reagent (see page 540) to each tube and heat in boiling water for eight minutes. Cool and add 2 c.c. of phosphomolybdic reagent (see page 540) to each tube. Dilute to 25 c.c. mark and compare in colorimeter.

6. Calculate as follows:

$$\frac{20}{R} \times 0.6 \times \frac{100}{0.01} \times \frac{1}{1000} = \text{per cent lactose} = \frac{120}{R} = \text{per cent lactose}$$

7. For the standard lactose solution, prepare a stock lactose solution by dissolving 1 gram of lactose (weighed on an analytical balance) in 0.2 per cent
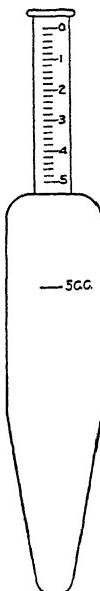
benzoic acid and making up to 100 c.c. in a volumetric flask. The working standard is prepared by diluting 3 c.c. of this stock solution in a 100 c.c. volumetric flask to the mark with 0.2 per cent benzoic acid. Two c.c. of this solution equal 0.6 milligram lactose.

CHEMICAL ANALYSES OF DIABETIC FOODS

Preparation of Sample.—Grind sufficiently fine in a mortar or mill to pass through a 1 millimeter sieve. Preserve in a sealed container to prevent moisture changes.

Detection of Starch.—Starch is best detected, when present to any appreciable extent in any mixture, by boiling a portion of the sample in water, cooling and applying a solution of iodine. A characteristic blue color appears if starch is present. Very small amounts of starch are best identified in powder on a microscope slide, or better, to the powder previously rubbed with water on a slide under a cover glass; the starch granules, if present, will be colored intensely blue by the iodine, and are at once rendered apparent when viewed through the microscope.

Estimation of Moisture.—Weigh accurately 2 to 3 grams of foodstuff on a tarred watch glass and dry to constant weight in an oven at 105° to 106° C. This requires about four hours. The loss in weight represents moisture.

Estimation of Ash.—Follow the procedure for determining the ash of human milk.

Estimation of Protein.—Determine the total nitrogen of a 1 gram sample according to the procedure given for the total nitrogen of milk. Calculate the protein by multiplying the total nitrogen by the appropriate factor which varies with different cereals as follows: wheat, 5.70; rye, 5.62; oats, 6.31; corn, 6.39; and barley, 5 82. If the sample represents a mixture of various grains the conventional factor 6.25 is employed.

Estimation of Fat.—Heat on a boiling water bath for ninety minutes 5 grams of the sample in a 200 c c. flask with 50 c.c. of water and 2 c.c. of 25 per cent hydrochloric acid (specific gravity 1.125). Cool, nearly neutralize with 40 per cent sodium hydroxide (using 0.04 per cent methyl orange as indicator) and filter. Transfer contents to a filter paper and wash with hot water. Dry filter and contents in oven at 105° C. and transfer to extraction thimble of Soxhlet extractor. Extract with ether for sixteen hours, transfer the extract to a weighed beaker, evaporate the ether on the water bath and dry to constant weight in the oven. This weight represents the fat.

Estimation of Starch, Sugar and Dextrin.—1. Extract 4 to 5 grams of the fine powder (accurately weighed) on a hardened filter paper with five successive 10 c.c. portions of ether.

2. Wash the powder into a beaker with 50 c.c. of water.

3. Immerse the beaker in a boiling water bath for fifteen minutes or until all the starch is gelatinized.

4. Cool to 55° C.

5. Add 20 c.c. of a fresh 0.5 per cent aqueous solution of U.S.P. pancreatin.

6. Digest at 55° C. for one hour.

7. Heat again to boiling for a few minutes to gelatinize the remaining starch granules.

8. Cool to 55° C. and redigest at this temperature with another 20 c.c. portion of pancreatin solution for one hour or until the residue treated with iodine gives no test for starch.

9. Cool, make up to 250° C. and filter.

10. Place 200 c.c. of the filtrate into a flask, add 20 c.c. of dilute hydrochloric acid (specific gravity 1.125; made by adding 2 volumes of water to 5 volumes of concentrated acid) connect with a reflux condenser and heat in a boiling water bath for two and a half hours.

11. Cool, nearly neutralize with 10 per cent sodium hydroxide, finish the neutralization with sodium carbonate and dilute to 500 c.c.

12. Mix the solution, pour through a dry filter and determine the dextrose in an aliquot part according to Benedict's method for sugar in urine.

13. CALCULATION: The amount of dextrose found multiplied by 0.9 gives the amount of starch. This amount multiplied by the aliquot part taken and the two dilutions gives the starch in the original sample from which the percentage may then be calculated.

CHAPTER XXXV

METHODS FOR TOXICOLOGICAL EXAMINATIONS

Principles.—1. It is highly important that a sufficient amount of material be furnished. Depending on the expected concentration of the substance, 500 to 2000 c.c. of urine or 100 to 200 grams of feces are desired. In cases of acute mercury poisoning with oliguria, small amounts of urine can be examined when more is not obtainable.

2. Material desirable for detection of some common poisons:

Lead: Feces, urine. Feces contains more than urine

Mercury: Urine, feces. Excretion about equal in the two. Urine is desired when obtainable, as analysis is more quickly and easily done.

Arsenic: Urine. Some in feces

Morphine: Feces. Urine

Methyl alcohol: Urine. Examination for formic acid

REINSCH METHOD FOR ARSENIC, ANTIMONY, AND MERCURY

The Reinsch test possesses two advantages: (1) as a preliminary test when abundance of material is available and (2) for clinical purposes during the life of the patient. It is a test not only for arsenic, but for mercury and antimony as well. It may be applied directly to a liquid containing organic matter, as the urine, and may be completed in a few minutes.

If a solution containing an arsenite and acidulated with about one-fifth its volume of hydrochloric acid (arsenic free) be heated a little below the temperature of boiling water in the presence of metallic copper foil (arsenic free), a gray stain is formed upon the copper which is an alloy of copper and arsenic. It is not formed in the presence of powerful oxidizing agents such as the chlorates. With an arsenate it is only slowly formed. If the presence of arsenates be suspected, it is well to reduce them to arsenites by sulphur dioxide and expel the excess of gas by boiling before applying the test. A stain having an appearance similar to that caused by arsenic is also formed if the liquid contains compounds of sulphur, selenium, gold, platinum, silver, bismuth, antimony or mercury.

To distinguish the arsenical stain from the others the strip of thin copper foil, which should be about one-eighth by three-quarters of an inch, is taken from the solution, gently washed and dried by contact with filter paper. It is

then inserted into one end of a clean piece of thin glass tubing, open at both ends and about 8 inches long. This is held at an angle of about 10 degrees to the horizontal and gently warmed along its entire length until the interior of the tube and the foil are perfectly dry. The portion of the tube immediately above the copper is then slightly warmed (to insure the formation of larger crystals than would be deposited upon a cold surface) and then, the forefinger being more or less applied to the upper opening in such a manner as to allow a very slow current of air to go through the tube, the copper is heated strongly. There is danger of loss if the air current is too rapid.

Of the compounds mentioned, selenium, arsenic, antimony and mercury are the only ones which produce a sublimate in the tube. Sulphur is volatilized as sulphur dioxide, and the metals remain upon the copper.

The sublimate produced by mercury is grayish rather than pure white, and, when examined with the microscope, is found to consist of an aggregation of shining globules. The deposits of the oxides of arsenic, antimony and selenium are white and more closely resemble each other, but differ in certain particulars. The *antimonial deposit* is nearer to the point at which heat was applied than the arsenical, and a portion of it may be in that part of the tube which was in the flame After the formation of the sublimate it may be readily driven along the tube by a moderate heating if it be *arsenic,* while much higher temperature is required to volatilize the antimonial deposit. The arsenical deposit consists entirely of brilliant octahedral crystals varying in size, the larger being in the portion of the sublimate nearest to where heat was applied. The crystals are bright, with finely defined edges, and scintillate when the tube is rotated on its axis in the sunlight. The *antimonial deposit* is generally entirely amorphous. It may, however, contain crystals some of which may be octahedral of the same shape as the arsenical crystals, but rather duller in luster and less transparent, and whose edges appear as broader black lines. These crystals, if present at all, are always few in number and are surrounded by much granular material, and require a high temperature for their volatilization. Occasionally prismatic crystals are also formed either beyond the copper or in that part of the tube which was in the flame.

The presence of *selenium* is exceptional, originating most frequently as an impurity of sulphuric acid. Microscopically its sublimate is found to consist of amorphous material and may contain prismatic crystals arranged in feathery bundles.

Two points are to be borne in mind: Hydrochloric acid is rarely free from arsenic and the copper foil may contain it. The method should, therefore, never be used without a blank. If the chemicals be pure the copper is, if anything, brightened. Should it become dimmed in the slightest degree, the acid, which is usually at fault, must be rejected. An objection to this method is the fact that copper is introduced into the articles under examination. It should therefore never be used except with a small sample of the available material.

The practical limit of delicacy of this test is about 0.0065 milligram. It is certainly inferior in delicacy to the Marsh test.

It should also be remembered that normal urine may and frequently does, contain arsenic. The amount of this so-called normal arsenic is variable, depending upon food, occupation and environment. The Reinsch test is hardly sufficiently delicate to detect this "normal" arsenic, but the Marsh test usually will give a test for arsenic with normal urine.

DETERMINATION OF LEAD

Preliminary Treatment: Feces and Tissues.—1. Free from water by heating in a porcelain dish (Coors).

2. After the material begins to char, bring to a dull red heat and ash. Ashing must be very carefully conducted at a temperature well below full red heat, otherwise part or all of the lead will be lost by volatilization. Fecal material usually ashes readily, but the tissues form a residue which must be repeatedly extracted before the entire char is consumed. Usually the material requires re-ashing as a certain quantity of inorganic salts fuse and prevent complete oxidation.

3. After the first ashing the material should be cooled and dissolved with dilute hydrochloric acid and hot water. It is essential that *all* the ash be dissolved, for frequently lead phosphate is present as an insoluble residue which may be mistaken for silica. If this residue is insoluble in hydrochloric acid it should be treated with a mixture of hydrochloric and tartaric acids (which dissolves lead phosphate), until the ash is quantitatively dissolved. (Tartaric acid even of good quality usually contains lead and, therefore, should be tested with hydrogen sulphide.)

Urine.—1. Ammonium hydroxide is added to urine until it is strongly ammoniacal. This mixture is allowed to stand one to twenty-four hours. In this reaction the earthy phosphates are precipitated and lead phosphate is carried down.

2. The gelatinous mass of phosphates settles into a compact mass from which the clear lead-free liquor may be decanted and the remainder rapidly filtered by suction on a Buchner funnel.

3. The filter paper containing the precipitate is ashed and the precipitate completely dissolved in dilute hydrochloric acid with the aid of heat if necessary. (The urine must be either freshly collected or well preserved with thymol, because crystalline phosphates which form when urine is allowed to become ammoniacal on standing, do not completely remove the lead. Heating ammoniacal urine to increase the rate of settling of phosphates prevents complete recovery of lead.)

Procedure.—1. Carefully neutralize the solution with dilute sodium hydroxide using methyl orange as indicator. Add dilute hydrochloric acid until the solution is just acid to methyl orange.

2. Saturate the cold solution with washed hydrogen sulphide. If sulphides precipitate to any great extent during this process, they may be separated at once, but if no precipitate appears, the solution, saturated with hydrogen sulphide, should be allowed to stand overnight. Centrifuge and wash with boiled distilled water, three times altogether. (FeS is easily oxidized by the air to soluble $FeSO_4$.)

3. Dissolve the precipitate in nitric acid (3 to 5 c.c. concentrated). Boil to expel hydrogen sulphide, cool, and neutralize with dilute sodium hydroxide, using phenolphthalein as indicator.

4. Acidify with dilute acetic acid, and add an excess of potassium chromate —2 or 3 drops of a saturated solution. If the solution is held against a dark background during this process a slight turbidity may be observed around the drop of added chromate in the presence of even very minute quantities of lead. To hasten the reaction the solution should be boiled for a few minutes. If no turbidity is apparent the solution should be allowed to stand overnight. A yellow precipitate indicates the presence of lead.

5. If quantitative estimation is desired, proceed as follows: Filter; wash with warm water to remove all soluble chromate from the filter paper, and wash the precipitate completely into an Erlenmeyer flask. Wash the filter paper with 2 to 5 c c. of 1:1 solution of hydrochloric acid followed by warm water, and collect in the same flask. The precipitate dissolves readily in hydrochloric acid. Add an excess of potassium iodide solution at once and titrate the liberated iodine with N/200 sodium thiosulphate, a drop or two of starch being added near the end-point as indicator:

$$1 \text{ c.c. } N/200 \text{ sodium thiosulphate} = 0.345 \text{ milligram lead}$$

When only small amounts of lead (less than 1 milligram) are present, use a micro-buret graduated in 0 2 c.c. The sodium thiosulphate should be made up and preserved with suitable precautions for prevention of decomposition by carbon dioxide. Restandardize once a week.

DETECTION OF METALLIC POISONS

Oxidation of Organic Matter (Fresinius von Babo's Method).—1. A portion of finely divided material is mixed with distilled water to a fluid mass and placed in a Kjeldahl flask.

2. About 30 c.c. of concentrated hydrochloric acid (arsenic-free if a test is to be made for arsenic) are added per 100 c.c. of material (a large excess of hydrochloric acid should be avoided).

3. Add 1 to 2 grams of potassium chlorate, shake well and set the flask upon a boiling water bath under the hood. Nascent chlorine is evolved which destroys the organic matter. When the flask is hot, it is frequently shaken and a trace of potassium chlorate (0.3 to 0.5 gram) is added from time to

time until the solution is a pale yellow color and longer heating produces no further charge. Fat is very resistant to oxidation by chlorine.

4. When oxidation is complete, dilute with hot water and add a few drops of dilute sulphuric acid to precipitate possible barium; shake and pour the liquid through a wet filter paper.

5. Evaporate in a porcelain dish on a water bath nearly to dryness to remove excess acid. The decomposition of some potassium chlorate may give a brown color at this point. (If necessary, filter, wash with water, and evaporate again almost to dryness.)

6. Dissolve in water and filter. (The insoluble residues may contain silver chloride, barium sulphate and lead sulphate in addition to fat. These can be identified if necessary, after fusion with potassium nitrate and sodium carbonate.)

Treatment with Hydrogen Sulphide.—The filtrate should have only a faint yellow color, and should be slightly acid (test with litmus). Place in a flask and heat on a water bath. While heating saturate the solution with washed hydrogen sulphide from a Kipp generator. (If a test is to be made for arsenic, the hydrogen sulphide must be arsenic free. Prepare arsenic-free hydrogen sulphide by saturating dilute sodium hydroxide solution with hydrogen sulphide from crude iron sulphide and commercial hydrochloric acid.) Pour this sodium hydrosulphide (NaSH) solution into a separating funnel and add slowly to dilute (1:4) sulphuric acid. Pass hydrogen sulphide into the hot solution for thirty minutes and continue for about thirty minutes after the flask has cooled, then stopper tightly and let stand for several hours, preferably overnight, and filter. The filtrate may contain chromium or zinc. The precipitate may contain arsenic, antimony, tin, mercury, lead, bismuth, copper, cadmium. Treatment with hydrogen sulphide almost invariably causes a precipitate of sulphuric and organic thio-compounds; therefore no positive conclusion can be drawn from the formation of precipitate at this stage.

Examination of the Precipitate.—The precipitate is thoroughly washed with hydrogen sulphide water and while still moist about 5 to 10 c.c. of a boiling solution of equal parts of ammonium hydroxide and yellow ammonium sulphide are dropped upon the precipitate on the filter. Repeat this several times. Finally wash with a few c.c. of a fresh mixture of ammonia and yellow ammonium sulphide. The filtrate may contain arsenic, antimony, tin, and copper (see Metallic Poisons I). The precipitate may contain mercury, lead, copper, bismuth, and cadmium (see Metallic Poisons II).

Metallic Poisons I.—Evaporate the solution (the solution is often dark brown owing to dissolved organic substances) to dryness in a porcelain dish on a water bath, cool, moisten with fuming or concentrated nitric acid and again evaporate to dryness. Then mix the residue with three times its volume of a mixture containing 2 parts sodium nitrate and 1 part sodium carbonate. Thoroughly dry this mixture upon the water bath and introduce small portions at a time into a porcelain crucible containing a little fused sodium nitrate

heated to redness. After the final addition heat the crucible a short time, introducing possibly a little more sodium nitrate until the fused mass is color-less. In the presence of copper the melt is gray or black from copper oxide. Sodium arsenate, sodium pyro-antimonate, sodium stannate as well as stannic oxide and copper oxide may be present. Soften the cold melt with hot water and wash into a flask. Add a little sodium bicarbonate to decompose the small quantity of sodium stannate possibly in solution and precipitate all the tin as stannic oxide and then filter. The filtrate contains any arsenic present as sodium arsenate and the residue may contain sodium pyro-antimonate, stannic and copper oxide.

Marsh Test for Arsenic.—Acidify the filtrate with arsenic-free sulphuric acid. Evaporate in a casserole over a free flame, and add sufficient arsenic-free
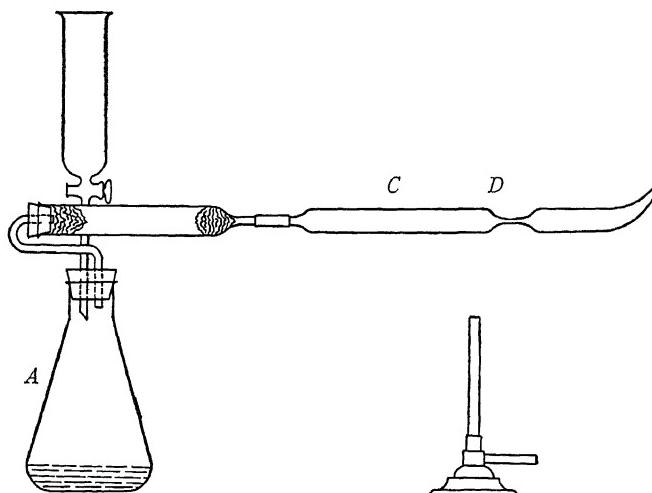
FIG 298 —ONE FORM OF MARSH APPARATUS USED IN TESTING FOR ARSENIC.

sulphuric acid to expel nitric acid   Heat until copious white fumes of sulphuric acid appear. Arsenic if present is in the form of arsenic acid and is tested in the Marsh apparatus (Fig. 298).

Place about 30 grams of arsenic-free granulated zinc in flask *A*. Since pure acid invariably will not react with pure zinc it is necessary to add about 100 milligrams of copper sulphate, thereby forming an electric couple and causing hydrogen to be formed as soon as acid is added. Stopper tightly and pour 50 c.c. cold, dilute, arsenic-free sulphuric acid (1 volume concentrated sulphuric acid: 5 volumes water) into the funnel and regulate the flow of this acid upon the metal so that the hydrogen will not be generated too violently. The flask should be kept cool during the analysis by keeping it sur-rounded with cool water. If the temperature gets too high, sulphur dioxide is formed and this in the presence of hydrogen is reduced to hydrogen sul-

phide (H_2S), which interferes with the test. All joints of the apparatus should be tight to avoid escape or arseniureted hydrogen (AsH_3) and also to prevent explosions. Air should be completely expelled before igniting to prevent explosion. To tell when this point is reached, collect hydrogen in a dry test tube until it ignites without detonation when carried to a flame. It may require ten minutes to expel the air. Test the hydrogen to insure its entire freedom from arsenic. Neither the arsenic mirror nor spot appear. With the hydrogen burning at the outlet, gradually introduce the perfectly cold sulphuric acid solution containing arsenic in small portions into the flask A by means of the same funnel. At the same time heat ignition tube C to redness just back of the constriction D If the solution contains arsenic, a shining metallic arsenic mirror is deposited just beyond the point of ignition. If the flame is removed from C and a cold porcelain dish pressed down on the arsenic hydrogen flame at the tip, a brownish black spot is formed upon the dish. This spot dissolves readily in sodium hypochlorite solution (Dakin's). Antimony spots will not dissolve.

Extinguish the flame at the end of tube C and hold in the escaping gas a strip of filter paper moistened with concentrated silver nitrate solution ($1 : 1$). A yellow stain appears if the hydrogen contains arsenic and a drop of water. (See note regarding presence of "normal arsenic" under Reinsch test.)

Metallic Poisons II.—Over the substance on the filter paper remaining after the treatment with the hot ammonium sulphide mixture there are poured several small amounts of warm, rather dilute nitric acid (1 volume concentrated nitric acid: 2 volumes water). Mercuric sulphide does not dissolve but all the other sulphides pass into solution as nitrates. The filtrate may contain lead, copper, bismuth, and cadmium, and specific tests are available for their recognition. (If the filtrate contains lead, dilute sulphuric acid produces a white precipitate of lead sulphate.)

TEST FOR MERCURY.—Treat the substance remaining on the filter paper after nitric acid treatment, even though not black, with a little hot dilute hydrochloric acid containing a crystal of potassium chlorate, and pass the acid through the paper several times. Evaporate the filtrate to dryness in a porcelain dish upon the water bath and dissolve in 5 c c. of 5 per cent hydrochloric acid, filter and test the filtrate for mercury as follows:

(*a*) *Stannous Chloride Test*—Add to a portion of the filtrate a few drops of stannous chloride solution. A white precipitate of mercurous chloride appears if mercury is present. Excess of stannous chloride, especially if heat is applied, reduces this precipitate to gray metallic mercury.

(*b*) *Copper Test*—Put a few copper slugs (previously cleaned in concentrated nitric acid and washed) into a portion of filtrate. Mercury deposits as a gray spot on standing or on heating. Wash the slugs successively in water, alcohol, and ether. Dry thoroughly and heat in a small bulb-tube of hard glass. Mercury sublimes and collects in small metallic globules on the

cold sides of the tube. A trace of iodine vapor introduced into the tube immediately transforms the gray sublimate into scarlet mercuric iodide.

The following outline shows the general plan of the above system of analysis.

Material:
Treated with hydrochloric acid and potassium chlorate. Dilute sulphuric acid. Filter.

Filtrate: Saturate with warm hydrogen sulphide.		Precipitate tested for silver, lead, barium.
Precipitate: Treated with hot ammonium sulphide and ammonium hydroxide.	Filtrate tested for chromium, zinc.	
Filtrate tested for arsenic, antimony, tin, copper (Metallic Poisons I.)	Precipitate tested for mercury, lead, bismuth, copper, cadmium. (Metallic Poisons II.)	

DETERMINATION OF CARBON MONOXIDE IN BLOOD

Principle.—A light brownish-gray suspension is formed when normal blood diluted with water is treated with a solution of pyrogallic and tannic acids. A light carmine suspension is formed with carbon monoxide hemoglobin.

Reagent.—PYROGALLIC-TANNIC ACID SOLUTION

Tannic acid, C.P. (2 per cent sol.).... ...... 25 c.c.
Pyrogallic acid, C.P. (2 per cent sol.). ...... 25 c.c.
To be made up fresh.

Preparation of Standards.—1. Procure about 10 c.c. of normal blood which has been prevented from clotting by the use of an anticoagulant (potassium or sodium citrate is the anticoagulant of choice) in the proportion of 0.05 gram per 10 c.c. of blood

2. Pipet 3 c.c. of blood into a 50 c.c. graduated cylinder and dilute with distilled water to the 30 c.c. mark.

3. Saturate the rest of the blood with 3 to 5 per cent carbon monoxide gas ("illuminating gas" may be used) and then dilute 3 c.c. to a volume of 30 c.c. with distilled water.

4. From these solutions of oxyhemoglobin and carbon monoxide hemoglobin, mixtures are made which vary from 0 to 100 per cent in steps of 10 as follows:

Per Cent	Carbon Monoxide Hemoglobin, c c.	Oxyhemoglobin, c.c.
100	2	0
90	4.5	0.5
80	4	1
70	3.5	1.5
60	3	2
50	1	1
40	2	3
30	1.5	3.5
20	1	4
10	0 5	4.5
0	0	2

Two c.c. amounts of these solutions are placed in small test tubes of uniform bore.

5. To each standard thus prepared are added 2 c.c. of strictly fresh pyrogallic-tannic acid solution and the tubes are inverted twice to insure mixing. Do not shake.

6. The tubes should be sealed immediately by pouring a little melted paraffin on top of the contents while the tube is immersed in cold water as a caution against overheating.

Procedure (Sayers and Yant).—1. Dilute 0.5 or 1 c.c. of the whole blood to be analyzed, prevented from clotting by the addition of an anticoagulant, 1 : 10 with distilled water.

2. Pipet 2 c.c. of this diluted blood into a test tube uniform in bore with those of the standards and add with a pipet 2 c.c. of strictly fresh pyrogallic-tannic acid solution. Invert the tube several times to insure mixing. Do not shake!

3. Let stand fifteen minutes and read against the standards. If carbon monoxide is present, let stand fifteen minutes longer, read and report the latter reading as "per cent blood saturation with carbon monoxide."

NOTES.—1. In carbon monoxide bloods prevented from clotting by oxalate, there is an appreciable change of carbon monoxide to carbon dioxide on standing. This is not true when citrate or sodium fluoride are used.

2. In order to prevent loss of blood due to excessive foaming, the operation should be carried out in a beaker with constant stirring.

3. The blood for the standards should be saturated with carbon monoxide before it is diluted with distilled water in order to minimize the volume of carbon monoxide gas physically dissolved in the resulting solution.

4. If it is desired to preserve the standards, air must be excluded. The tube walls above the paraffin should be thoroughly dried and a permanent seal made by placing a disk of cardboard on top of the paraffin and filling the remainder of the tube with ordinary sealing wax. Standards thus prepared and kept in a cool place will retain their permanence for one to two weeks,

not changing enough to interfere with the accuracy of the determination. Permanent standards prepared from artists' oil colors are on the market.

ESTIMATION OF FORMIC ACID IN URINE

Principle.—Carbohydrates are removed by copper hydroxide. Formic acid is separated by steam distillation and is concentrated as the sodium salt. Formic acid reduces mercuric chloride with the formation of mercurous chloride which can be determined gravimetrically.

Procedure.—1. One hundred c.c. of urine are accurately measured into a liter volumetric flask containing 500 to 600 c.c. of water.

2. One hundred c.c. of 20 per cent copper sulphate are added and the whole is well mixed.

3. A 10 per cent suspension of calcium hydroxide is then added until alkalinity, as evidenced by the characteristic color change from green to blue, occurs. It is well to avoid an excess of calcium hydroxide. The contents are then made up to volume and mixed.

4. After standing fifteen to thirty minutes they are filtered and a 600 c.c. aliquot is placed in an 800 c.c. Kjeldahl flask, with 2 to 3 drops of phenolphthalein as an indicator, and made distinctly acid with 85 per cent phosphoric acid.[1] Sufficient excess should be added to free all of the formic acid present (1 to 2 c.c. are ample for this purpose). Glass beads are added to prevent bumping.

5. This flask is connected to a supply of steam and to a water condenser by means of a Kjeldahl trap (Fig. 299). Both the neck of the flask and the outside of the trap are well wrapped with asbestos cord to prevent loss of heat.[2]

6. The distillate is caught in a casserole provided with 15 to 20 c.c. of N/10 sodium hydroxide, and a few drops of phenolphthalein are added to make sure that it is alkaline. This alkalinity must be maintained throughout the distillation by the addition of further N/10 sodium hydroxide as may be necessary, but a great excess must be avoided. It is neither necessary nor advisable to cause the delivery tube from the condenser to drop under the liquid in the casserole. The distillation must be so managed that at first a slow stream of steam is conducted in, while the contents of the distilling flask are rapidly driven over by brisk heating and reduced in volume to 50 or 75 c.c. At this amount they must remain until 2 liters are collected.[3]

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[1] Tartaric acid may be used instead of phosphoric, but we have not found that it has any particular advantage   Strong mineral acids cannot be used

[2] Such a device is essential, as otherwise phosphoric acid is mechanically carried over during the rapid distillation and causes much trouble. It is convenient to do the steam distillations on a Kjeldahl rack

[3] This reduction in volume is necessary because the amount of formic acid carried over by steam distillation varies enormously with the volume of the distilling fluid On the other hand, if the volume is allowed to become too low, especially if it boils dry, large errors result from the breaking down of other organic substances present

7. The distillate is then evaporated to dryness overnight on the water bath, and the residue taken up in exactly 100 c.c. of distilled water and filtered. A 90 c.c. aliquot is placed in a 250 c.c. Erlenmeyer flask and made just acid with N/10 hydrochloric acid. Ten c.c. of the special corrosive sublimate (HgCl₂) mixture [4] are then added and the flask is fitted with an air condenser and heated in a boiling water bath for one hour. After cooling, the mercurous chloride is filtered into a weighed Gooch crucible, washed with 100 c.c. of 5 per cent cold hydrochloric acid, then water, alcohol, and ether, and dried one hour at 105° F. and weighed. The blank of the reagents in our experiments
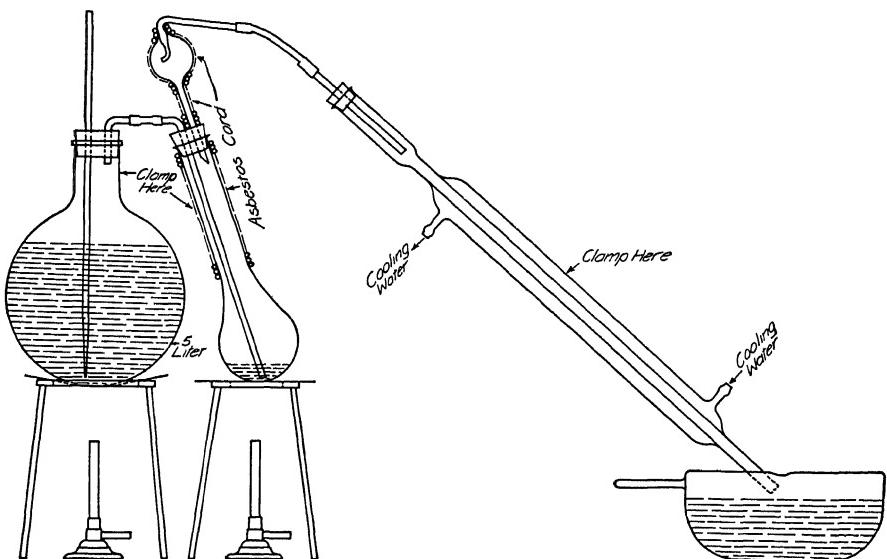
FIG. 299—APPARATUS FOR STEAM DISTILLATION.

has varied from 0.0014 to 0.0044 gram according to the reagent used. The amount of formic acid per liter in the original urine is then:

$$1.01 \times \frac{10}{6} \times \frac{10}{9} \times 0.0975 \times (\text{weight of precipitate—weight of blank of reagents})[5]$$

NOTES.—1. The addition of sodium chloride and sodium acetate to the mercuric chloride solution hastens very greatly the precipitation of mercurous chloride and also prevents the precipitation of impurities.

into formic acid. Two liters of distillate are sufficient to collect if these precautions are observed. It should be collected in about two hours.

[4] This solution contains 200 grams of corrosive mercuric chloride, 80 grams of sodium chloride, and 300 grams of sodium acetate to 1 liter of water. Ten c c. of this solution are sufficient for amounts of formic acid up to 0.13 gram, that is, a concentration sixteen times the upper limit of normal urine. For urines containing larger amounts, larger amounts of this mercuric chloride mixture must be used.

[5] One gram of mild mercurous chloride equals 0.0975 gram of formic acid.

2. Determinations of formic acid content of urines should be commenced as soon as possible after collection. Concordant results cannot be obtained when specimens stand over twenty-four hours even though they are kept on ice or have preservatives added. Errors ranged from 0 to 4.8 per cent in recovery experiments.

3. Formic acid content of normal urine from individuals on mixed diet has been found to vary between 29 9 and 118 6 milligrams per twenty-four hours.

4. Increase in consumption of carbohydrates causes increased formic acid excretion. An increased excretion of formic acid does not occur in the acidosis of diabetes or in the acidosis of starvation. The one pathological condition in which formic acid excretion has been found to play a predominant rôle is in the acidosis of methyl alcohol poisoning.

DETECTION OF METHANOL

Principle.—Methanol is oxidized by potassium permanganate to formaldehyde which is detected by the development of a violet color with a reduced rosaniline solution.

Reagents.—1. POTASSIUM PERMANGANATE.—Dissolve 3 grams of potassium permanganate in 100 c.c. of water previously distilled over potassium permanganate, and containing 15 c.c. of phosphoric acid. By using water previously distilled from potassium permanganate solution, this reagent can be kept for a long time.

2. OXALIC ACID SOLUTION.—Dissolve 5 grams of oxalic acid in a solution made by diluting 50 c.c. of sulphuric acid (1 84) to 100 c c.

3. SCHIFF'S REAGENT (modified).—Dissolve 0.2 gram of rosaniline (fuchsin), or an equivalent weight of its salt, in 120 c.c. of hot water, cool, and add this to a solution of 2 grams sodium bisulphite in 20 c c. of water. Finally add 2 c.c. of concentrated hydrochloric acid and dilute the whole to 200 c.c. This solution should become colorless or nearly so after standing. If it is protected from the air no deterioration results. A very pure rosaniline base was used in these tests, but a product of lesser purity may be used, giving a somewhat colored reagent, which is, nevertheless, satisfactory.

Procedure.—1. Pipet 2 c c. of sample into a test tube.

2. Add 5 drops of 85 per cent phosphoric acid.

3. Add 1 c.c. potassium permanganate solution.

4. Allow to stand ten minutes.

5. Add 1 c.c. of oxalic acid solution (clear brown).

6. Add 5 c.c. 10 per cent sulphuric acid (colorless).

7. Add 2 c.c. of the modified Schiff's reagent.

8. Mix thoroughly. If methanol is present, a violet color is developed. With traces the color is not developed for an hour. If a positive test is found,

glycerol or pectin may be present and the sample must be distilled and again tested.

NOTES.—The method is applicable to the colorimetric quantitative estimation of minute amounts of methanol in alcohol and it is recommended that 5 per cent solutions of alcohol be used. Using 5 per cent ethyl alcohol solutions, the standards recommended for comparison are o oo, 0.01, 0.05, 0.1, 0.2 and 0.4 per cent methanol. It is quite necessary that standard conditions prevail, namely, (1) 5 per cent alcohol; (2) the same kind and amount of reagents; (3) ten minutes for oxidation with potassium permanganate; (4) uniform temperature (room temperature is satisfactory); (5) length of time (twenty minutes) in making comparisons after the addition of the modified Schiff reagent.

COLOR DEVELOPED WITH VARIOUS AMOUNTS OF METHANOL IN ALCOHOL

Methanol, Per Cent by Volume	Ethyl Alcohol, Per Cent by Volume						
	1	5	10	25	50	75	95
0 00	0	0	0	0	0	0	0
0 0025	1	0	0	0	0	0	0
0 005	2	1	1	0	0	0	0
0 01	3	2	2	1	0	0	0
0 03	4	3	2	1	1	0	0
0 05	4	4	3	2	1	1	0
0 08	4	4	4	3	2	2	1
0 10	4	4	4	4	3	2	2

0 = no color   1 = very slight color   2 = slight color   3 = fair color   4 = good color

QUALITATIVE TEST FOR MORPHINE

Fabinyi reports on a color test for morphine originally devised by Radulescu as follows: To the solution to be tested add a few drops of dilute hydrochloric or sulphuric acid, then a very small quantity of sodium nitrite either in dry form or solution, and finally make alkaline with either ammonia or sodium or potassium hydroxide. In the presence of morphine a red color at once appears which is destroyed by acid and restored by alkali. When the solution is very dilute the color is more of a mahogany shade. In strong solutions a green color is produced on adding the sodium nitrite and before the alkali is added. The same test is reported on by Radulescu who tried it on 150 substances and found but one (a lettuce extract of doubtful purity) that gave a similar indication. He considers that the color probably depends on the formation of nitroxanthranol. This color is not extracted by chloroform, carbon disulphide or ether. The test has the great advantage of being applicable to many compounds without extracting the morphine in approximately pure form. It accurately distinguishes morphine from codeine and

dionin, which give no color with the test, but is not so dependable in the case of heroin as this substance gives a slight color almost identical with that of morphine in very dilute solution, so by this test alone one cannot be certain whether there is present only a minute quantity of morphine or a large amount of heroin. With equal amounts of the two the color from the morphine is very much more intense. This test also distinguishes morphine from apomorphine, which alkaloid gives a peculiar and characteristic reaction described under apomorphine

TEISCHMANN'S TEST FOR BLOOD STAINS

Principle.—The test depends on the fact that hematin is formed from the decomposition of the hemoglobin by heat, and secondly, that the hematin in solution in boiling glacial acetic acid unites with the chlorine of the salt to form chloride of hematin, which is soluble in boiling glacial acetic acid, crystallizing from this solvent on cooling.

Procedure.—If the material is a dried blood stain, a small fragment of the dried blood should be removed from the stain with the point of a knife and transferred to a glass slide. If the stain be a diffused one, or if the blood, while still fresh, has soaked into the fabric, as in the case of a stain on cotton or linen cloth, then it suffices to scrape a small portion of the stain with the knife point, collecting the dust thus removed on a glass slide. The fragment of dried blood or the dust should then be treated on the slide with a small drop of water in which has been dissolved a minute fragment of sodium chloride. This drop should then be evaporated to dryness by gentle heat, the dried residue covered with a cover glass, a drop of glacial acetic acid allowed to run under the cover glass, and the slide again gently heated until bubbles of gas are seen to form in the liquid under the cover glass. This shows that the glacial acetic acid has been heated to the boiling point. If, now, the slide be allowed to cool, the microscope will reveal the characteristic crystals of chloride of hematin in case the stain examined contained blood. These crystals of chloride of hematin are called "hemin" crystals, and they have a characteristic form (Fig. 300).

The normal hemin crystals have a yellow to chocolate-brown color and separate in the form of small rhombic plates. They naturally vary a little in size according to the rapidity of their formation. Sometimes, particularly if the fragment of dried blood on the slide was of considerable size, the form of the crystals in some parts of the preparation may be somewhat modified, some assuming a pointed, oval shape, and in some the outlines may be a little irregular; in all cases, however, a sufficient number of the normal perfect crystals will be seen to render their identification positive.

PRECAUTIONS.—1. Care should be taken in heating the slide not to raise the temperature so high as to decompose the hematin in the first dry residue obtained   If the temperature be raised to about 142° C. (287.6° F.), no hemin crystals will be formed.

2. On further heating the slide, after the addition of glacial acetic acid, the temperature should not be raised so high as to produce active boiling of the acid, since active ebullition may carry all the pigment beyond the edge of the cover glass, which might prevent the detection of the hemin crystals.

3. The hemin test will not detect blood pigment in blood stains that have been heated to a high temperature, that have been subjected to the prolonged action of naphtha, solution of ammonium chloride, or bromochloralum, or that have been exposed for a long time to direct sunlight.

4. This is by far the most important test for blood pigment, and it is extremely delicate. While the detection of hemoglobin shows with certainty
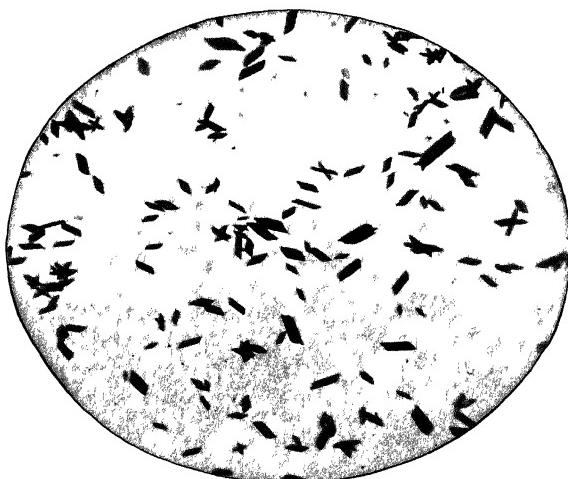
FIG 300—HEMIN CRYSTALS   (Wood)

the presence of blood, it throws no light upon the nature of the animal from which the blood came. To determine this latter question resort is had to microscopic examination of the blood cells and especially to the complement-fixation and precipitin tests.

CHAPTER XXXVI

METHODS FOR THE DIAGNOSIS OF EARLY PREGNANCY

Principles.—During pregnancy there is an increase in the amount of *anterior pituitary hormone* excreted in the urine. When urine containing an increased amount of this hormone is injected into female animals, such as mice, rats or rabbits, it produces marked changes in the ovaries. The changes are not observed in animals injected with urine from non-pregnant women. The tests are stated to be reliable after the tenth day following the first missed menstrual period following suspected conception.

The Aschheim-Zondek Test.—1. Collect morning specimen of urine in clean container. (Sterile precautions are not necessary.)

2. Adjust reaction to slightly acid if specimen is alkaline.

3. Filter through paper or centrifuge and use supernatant urine.

4. Mice or rats can be employed. If mice are used they should be young females weighing between 5 and 8 grams. Inject five animals under the skin of the back with the following amounts of urine:

Mouse	Dose
No. 1	0 2 c c.
No 2	0 25 c c.
No 3	0 3 c c.
No 4	. 0 35 c c.
No. 5	0 4 c c.

5. If rats are used they should be young females, thirty to forty-five days old and not weighing over 65 grams. Only one rat is used for each test. Inject subcutaneously with 0.5 c.c. of urine twice daily on three successive days.

6. On the fifth day following injection (the first injection in the case of the rat) anesthetize the animal and examine the genital organs before removal. Remove the organs and kill the animal by bleeding or with chloroform.

7. In positive cases the ovaries are enlarged, hyperemic and show hemorrhagic spots and yellowish protrusions which are due to hemorrhages into the follicles and corpora lutea respectively. The uterine horns are enlarged, the hymen open and smears of the vaginal secretion show changes in the cell types due to estrum. These vaginal changes are due to *female sex hormone* and therefore alone can not be considered as positive findings. Histological examination of sections of the ovaries may be helpful.

PLATE XI
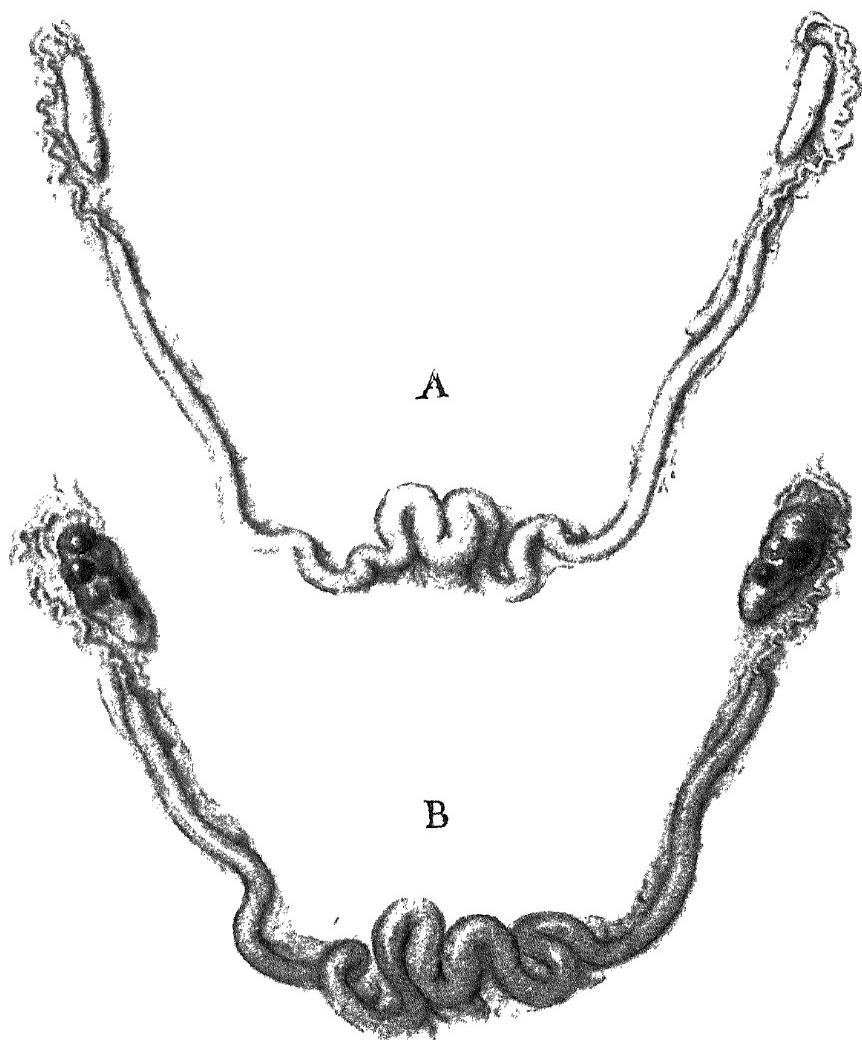
A Hormone Test for the Diagnosis of Early Pregnancy

A Bicornate uterus, tubes, and ovaries of a fourteen-week rabbit, thirty hours after injection of 7 c c. of urine from a non-pregnant patient.   This demonstrates a negative result, with no changes occurring in the ovaries

B   Bicornate uterus, tubes, and ovaries of a fourteen-week rabbit, thirty hours after injection of 7 c c of urine from a pregnant patient   This demonstrates a positive result from an approximate five weeks' pregnancy, showing the presence of numerous corpora lutea and corpora hemorrhagica

(From P. F Schneider, *Surg , Gynec & Obst ,* Jan 1931)

The Friedman Test.—1. Collect morning specimen in clean container.

2 Filter

3 Inject 5 to 7 c c in the ear vein of a female rabbit which should be from twelve to fourteen weeks of age   Sterile precautions are not necessary

4 Kill the rabbit in from twenty-four to thirty hours after injection and autopsy immediately

5 If negative, the ovaries remain small in size and show no change (see *A* in Plate XI) ; if positive, from one to fourteen corpora haemorrhagica and corpora lutea are found present in each ovary   In some instances, a positive result has been obtained in twelve hours, and Schneider recommends injecting two rabbits in cases in which a diagnosis of pregnancy might influence a decision regarding operation   The first rabbit is then autopsied at twelve hours, and the result checked by autopsy on the second rabbit at twenty-four or thirty hours without loss of time.

CHAPTER XXXVII

METHODS FOR THE MICROSCOPIC EXAMINATION OF TISSUES

By Wm. C. MacCarty, M.D.

and

W. L. A. Wellbrook, M.D.

PREPARATION OF TEMPORARY FROZEN SECTIONS OF FRESH TISSUE

1. Fresh tissue (the cells must be alive or, if not, kept in the ice chest, not more than thirty minutes out of the living body) in blocks not more than 2 by 10 by 10 millimeters is frozen on a good microtome and cut in sections 5 to 15 micra thick. Very small pieces may be blocked on stage in a few drops of water or dextrose solution and cut.

2. Remove the sections from the knife with the tip of the finger and allow them to thaw thereon, thus avoiding later development of air bubbles within sections.

3. Unroll the sections in 1 per cent sodium chloride solution.

4. Stain five to twenty seconds in concentrated Unna's polychrome methylene blue (Grubler's or Terry's modified and improved polychrome).

5. Wash out momentarily in 1 per cent sodium chloride solution.

6. Mount in Brun's glucose medium (glucose, 240 c.c.; distilled water, 840 c.c.; spirit of camphor, 60 c.c.; glycerin, 60 c.c.). Filter.

The Spencer automatic microtome with carbon dioxide attachment and vulcanite insulating plate gives excellent sections. Handle the sections from first salt solution through to the slide with a small glass rod lifter. Keep the sections constantly moving while in the stain. The smaller the stain cup the more readily tissue may be found if accidentally dropped from the lifter. If fluids for the unstained sections are in clear glass over a black background and those for stained sections are in white porcelain or clear glass over a white background, the work will be much facilitated. The various tissue elements are sharply contrasted in red, purple, and dark blue. Even mitotic figures, when present, are many times beautifully shown. Properly prepared fresh frozen sections reveal everything that may be seen by other methods without cellular distortions. The sections, of course, fade in from one to ten hours. Formaldehyde-fixed material, first thoroughly washed in tap water, may be treated in exactly the same manner as fresh tissue. The color differentiation is materially dif-

ferent but the process is of great convenience for the rapid examination of a great number of blocks from a given specimen.

PREPARATION OF PERMANENT FROZEN SECTIONS OF TISSUE

Reagents.—1. EHRLICH'S STOCK HEMATOXYLIN (A).

Hematoxylin crystals..................... 2 gm.
Water (distilled)......................... 100 c.c.
Alcohol (abs. or 95 per cent, C.P.).......... 100 c.c.
Glycerin................................. 100 c.c.
Glacial acetic acid....................... 20 c.c.

Add aluminum ammonium sulphate (about two handfuls). Ripen thirty to ninety days.

2. EHRLICH'S STOCK HEMATOXYLIN (diluted for use).

Stock solution (A)........................ 20 c.c.
Aluminum ammonium sulphate (sat. sol.).... 40 c.c.
Water (distilled)......................... 80 c.c.

3. CELLOIDIN.—Wash celloidin several times in 95 per cent alcohol; dry overnight on filter paper in 37° C. oven.

Celloidin................................ 12 gm.
Ether................................... 100 c.c.
Alcohol (95 per cent).................... 100 c.c.

Dilute for use by adding equal parts of ether and 95 per cent alcohol to small amount of celloidin.

4. STOCK EOSIN SOLUTION.

Eosin (aq. sol.)......................... 0.5 gm.
Water (distilled)........................ 100 c.c.

5. CARBOXYLOL.

Xylol................................... 300 c.c.
Carbolic acid (pure).................... 100 c.c.

Liquefy carbolic acid crystals by setting bottle in hot water.

6. ORTH'S DICHROMATE FIXING SOLUTION.

(*a*) Potassium dichromate................ 250 gm.
    Water (distilled).................... 6480 c.c.

(*b*) Sodium chloride..................... 108 gm.
    Formalin (40 per cent).............. 1200 c.c.
    Water............................... 3024 c.c.

Take 21 c.c. of (*a*) and 9 c.c. of (*b*) and mix as usual just before using.

7. FORMALIN (10 per cent) FOR FIXATION.—Must be used for frozen section cutting. Can be used for hematoxylin and eosin, Gram, Bielschowsky,

Weigert's iron hematoxylin and thionin stains; also for fat and Nile blue stains.

Procedure.—1. Cut section from tissue fixed in 10 per cent formalin or some other aqueous fixative for twenty-four hours.

2. Mount on slide from distilled water; wipe dry around section.

3. Drop 95 per cent alcohol (several drops) on section.

4. Drop of very thin celloidin (1 drop) on section.

5. Stain slide in hematoxylin for five minutes.

6. Wash slide through two glasses of tap water; leave in last glass five minutes. Use Coplin staining jars.

7. Dip slide in eosin.

8. Dip slide in distilled water.

9. Dip slide in alcohol (50 per cent).

10. Dip slide in alcohol (70 per cent).

11. Dip slide in alcohol (80 per cent); leave in two minutes.

12. Dip slide in alcohol (95 per cent) in two jars; leave in last jar two minutes.

13. Dip slide in carboxylol, in two jars; leave in last jar five minutes.

14. Dip slide in xylol, in three jars; leave in last jar two minutes.

15. Mount with Canada balsam.

16. Bone may be decalcified as follows and cut as ordinary tissue:

> Nitric acid .... 10 c.c.
> Water (distilled)..... . . . . 90 c.c.

Change daily until the tissue is soft. Leave in running water for several hours (or overnight).

PREPARING AND STAINING OF PARAFFIN SECTIONS

Procedure.—1. Place section or block of tissue in fixative solution for about eight hours; to hasten, place bottle containing section in incubator at 37° C. for four to five hours.

2. Place section in same bottle with 95 per cent alcohol for one to two hours, or stand overnight.

3. Place section in acetone for six hours or overnight in same bottle.

4. Place in equal parts of toluol and paraffin (melted in oven) for one and one-half hours at 57° C.

5. Pour off toluol and paraffin and save for use again (stopper). Place section in melted paraffin for two hours at 57° C. or may stand overnight at 37° C.

6. Imbed section in paraffin in watch glass which has been coated with glycerin. Float watch glass in cold water to harden.

7. Make squared block of hardened paraffin containing tissue.

8. Prepare clean slide by smearing a drop of egg albumin emulsion on it; then flood slide with distilled water.

9. Place paraffin block parallel with microtome knife and cut.

10. Cut section in ribbons 10 micra in thickness for routine work.

11. Float ribbon sections on water on slide. Heat gently to straighten but avoid melting paraffin.

12. Place sections in middle of slide and drain off water.

13. Place slide in slide box and place box in incubator at 37° C. for twelve hours or longer.

14. Place slide in xylol solution for two minutes until paraffin dissolves.

15. Place in acetone one minute.

16. Place in 95 per cent alcohol one minute.

17. Place in 80 per cent alcohol one minute.

18. Place in chloral hydrate 1 per cent, one minute; then place in tap water.

19. Place in hematoxylin (the same as used for frozen sections).

20. Wash in tap water.

21. Dip in 1 per cent acid alcohol (70 per cent).

22. Wash in tap or distilled water.

23. Place in saturated aqueous solution of lithium carbonate.

24. Wash in tap water.

25. Place in eosin solution for one minute (0.1 per cent aqueous eosin).

26. Dip in 95 per cent alcohol and then 80 per cent if necessary.

27. Wash well in acetone.

28. Place in carboxylol five to ten minutes (425 c.c. carbolic acid + 1275 c.c. xylol).

29. Place in xylol two to three minutes to remove carbolic acid.

30. Place in another dish of xylol for cleaning.

31. Mount with Canada balsam.

NOTES.—The active coloring agent in hematoxylin stains is hematin. The selective staining power of alum hematoxylin is due to the combination of this hematin with aluminum. The resulting blue color is precipitated in the tissues (chiefly the nuclei) by certain organic and inorganic salts there present. Hematin is formed from the hematoxylin by oxidation. The degree of blueness depends largely upon the freshness of the alum. In old, long-standing solutions sulphuric acid is formed and causes the solution to assume a reddish tint. There is a continuous formation of a precipitate due to the further oxidation of the hematin, in consequence of which it is always necessary to filter alum hematoxylin. As alum hematoxylin stains become older they stain more quickly but also more diffusely.

DECALCIFYING BONE FOR PARAFFIN SECTIONS

Procedure.—1. Place in 5 per cent nitric acid; change daily until it becomes soft (one to five days).

2. Wash in running water twenty-four hours.

3. Run up through alcohols, 80 to 95 per cent to absolute.

4. Place in cedar-wood oil for clearing until it becomes transparent and sinks.

5. Place in xylol for thirty minutes.

6. Place in paraffin in oven for two hours.

7. Embed in paraffin.

8. Cut sections as usual.

9. Stain as usual but preferably with eosin-methylene blue.

In place of steps 1 and 2, the following decalcifying fluid may be used (100 times volume of tissue should be used):

Hydrochloric acid........................ 4 c.c.
Glacial acetic acid............. .... 3 c.c.
Chloroform................... .. .. 10 c.c.
Water......................... ... . 10 c.c.
Alcohol (95 per cent)......... .... 73 c.c.

Pass tissue immediately into fresh absolute alcohol (two changes), then into cedar-wood oil. Another decalcifying fluid is as follows:

Formalin....... .... 1 part
Nitric acid.. .... 1 part
Water................................ 9 parts

This is a rapid method of decalcifying. Wash in running water as usual and then start in 80 per cent alcohol and run through a little slower.

PREPARING AND STAINING OF CELLOIDIN SECTIONS

Procedure.—This method is especially used in making sections of the eye.

1. Fix tissue in 10 per cent formalin and cut block.

2. Place it in 50 per cent alcohol for twenty-four hours.

3. Place in 70 per cent alcohol for twenty-four hours.

4. Place in 80 per cent alcohol for twenty-four hours.

5. Place in 95 per cent alcohol for twenty-four hours.

6. Then place in absolute alcohol for twenty-four hours.

7. Place in equal parts of absolute alcohol and ether for twenty-four hours.

8. Place in thin celloidin 3 per cent for from twenty-four to forty-eight hours.

THIN CELLOIDIN

Dry celloidin........................... 12 gm.
Ether.................................. 200 c.c.
Alcohol (abs.) .... 200 c.c.
Shake until all celloidin is dissolved.

9. Place in thick celloidin 6 per cent to 12 per cent for from twenty-four to forty-eight hours.

THICK CELLOIDIN

Dry celloidin............................ 30 gm.
Ether........   . . .. 225 c.c.
Alcohol (abs.)..... . ... 225 c.c.
Shake until dissolved.

10. Place tissue in thick celloidin in small paper box and under bell jar with concentrated sulphuric acid in a dish until firm.

11. Remove sulphuric acid and put in chloroform and let stand twenty-four hours.

12. Trim extra celloidin off around tissue but do not trim too close.

13. Put celloidin block in equal parts of chloroform and cedar oil for forty-eight hours. Add a few grains of copper sulphate.

14. Mount on fiber or wooden block and place block in cedar-wood oil for two days.

15. Cut sections about 15 micra thick.

16. Place sections in 80 per cent alcohol until ready to stain (until oil is removed).

17. Stain. (See directions for staining paraffin sections.)

18. Mount stained sections on slide.

VAN GIESON STAIN FOR CONNECTIVE TISSUE

Procedure.—1. Section from block of tissue out of any fixative and run through as usual to water.

2. Place in alum hematoxylin for four to ten minutes.

ALUM HEMATOXYLIN

(a) Hematoxylin......................... 1 gm.
    Alcohol (95 per cent).................. 100 c.c.
        Ripen for one week.

(b) Liquid ferrous sesquichlorate............ 4 c.c.
    Hydrochloric acid...................... 1 c.c.
    Water (distilled)....................... 95 c.c.
        Ripen for one week.
Mix equal parts of (a) and (b) and mix fresh before using.

3. Wash in distilled water.

4. Place in picric-acid fuchsin one minute.

PICRIC-ACID FUCHSIN

Picric acid (sat. aq. sol.).................... 20 c.c.
Acid fuchsin (1 per cent)................... 15 c.c.
Fibrous tissue stains pink, muscle yellow.

5. Wash in 95 per cent alcohol.
6. Wash in acetone.
7. Wash in carboxylol.
8. Wash in first xylol.
9. Wash in second xylol.
10. Mount with Canada balsam.

SCHARLACH R FAT STAIN

Procedure.—1. Fix tissue in 10 per cent formalin.
2. Cut frozen sections.
3. Wash section in distilled water.
4. Stain tissue in Harris' hematoxylin for one minute.
5. Wash in water.
6. Place in lithium carbonate for one to two minutes.
7. Wash in water.
8. Wash in 70 per cent alcohol.
9. Place in scharlach r for 10 minutes.

SCHARLACH R STAIN

Scharlach r	1 gm.
Alcohol (70 per cent made from abs) . . 100 c.c.	
Acetone.... .... 100 c.c.	

Place at 60° C. overnight.

Sudan III is made and used in same manner.

10. Wash quickly in 70 per cent alcohol.
11. Wash in distilled water.
12. Mount with glycerin or gum arabic.

GUM ARABIC SOLUTION

Gum arabic	40 gm.
Water (distilled).... . .. . 120 c.c.	

Dissolve by aid of heat on water bath; filter and add few thymol crystals.

STAINING TUBERCLE BACILLI IN TISSUES

Procedure.—1. Imbed in paraffin or use fixed frozen sections and cut sections 5 micra.
2. Run down to water in usual manner.
3. Place in hot carbolfuchsin and steam five to ten minutes.
4. Decolorize in 25 per cent hydrochloric acid.

5. Wash in distilled water.
6. Blot.
7. Place in Löffler's methylene blue for one minute.
8. Wash in water.
9. Blot.
10. Wash in 96 per cent alcohol.
11. Wash in aniline oil.
12. Wash in mixture of ⅓ anilin oil and ⅔ xylol.
13. Wash in xylol.
14. Mount with Canada balsam.

KLINGMULLER'S MODIFICATION OF ZIEHL-NEELSEN STAIN FOR TUBERCLE BACILLI IN TISSUES

Procedure.—1. Stain paraffin sections in carbolfuchsin for two to four hours at room temperature.

2. Wash in tap water.

3. Place in 5 per cent sulphuric acid until sections are pale pink.

4. Place in 96 per cent alcohol a few seconds—as long as the red washes out.

5. Counterstain with Löffler's methylene blue a few seconds so as to get a light blue tinge.

6. Wash in absolute alcohol.

7. Wash in xylol.

8. Mount in balsam.

NOTE.—When hydrochloric acid is used, the stained bacteria often disappear in a few days. Klingmuller's method, which substitutes sulphuric for hydrochloric acid, helps overcome this objection.

ANILINE GENTIAN VIOLET GRAM STAIN FOR BACTERIA IN TISSUES

Procedure.—1. Stain in gentian violet one minute. Gentian violet stain:[1]

STOCK SOLUTION

Gentian violet........................... 5 gm.
Alcohol (95 per cent)...... .... 50 c.c.

For use take 8 c.c. of stock solution to 42 c.c. of aniline water (5 c.c. aniline oil in 95 c.c. distilled water).

2. Wash in tap water.

3. Place in Lugol's solution one minute.

---

[1] Grubler's stain preferred.

LUGOL'S SOLUTION

Iodine.................................	1 gm.
Potassium iodide.....................	2 gm.
Water (distilled)...	200 c.c.

4. Wash well in tap water and blot.

5. Place in 95 per cent alcohol for a few seconds.

6. Place in aniline oil and xylol (equal parts) until nearly all the color is removed.

7. Clear in xylol.

8. Mount in balsam.

STAINING OF IRON IN TISSUES

Procedure.—1. Make paraffin or fixed frozen section as usual.

2. Run down to water as usual.

3. Stain in warm potassium ferrocyanide 2 per cent fresh solution (double distilled water) for three minutes until a blue color appears. Heat in beaker but not enough to turn color.

4. Wash in several changes of double distilled water.

5. Stain ten to thirty minutes in fuchsin:

Fuchsin................................	0.5 gm.
Alcohol (95 per cent)...	50.0 c.c.
Water.............................	50.0 c.c.

Or 0.5 per cent basic fuchsin in 50 per cent alcohol for ten to twenty minutes.

6. Differentiate and dehydrate in 95 per cent alcohol.

7. Wash in absolute alcohol.

8. Wash in xylol.

9. Mount with balsam.

WEIGERT'S MYELIN STAIN

Procedure.—Use formalin imbedded in celloidin. After cutting:

1. Wash in water.

2. Mordant twelve to twenty-four hours:

Potassium bichromate..................	2.0 gm.
Fluorchrome..........................	2.5 gm.
Water................................	100 c.c.

3. Wash in water two to three hours (few minutes to one to twelve hours).

4. Wash in 70 per cent alcohol for ten to fifteen minutes.

5. Wash in water ten to fifteen minutes to twelve hours.

6. Immerse in copper acetate one half saturated solution one hour (filter until blue).

7. Wash in water a few minutes to remove copper.
8. Stain two hours:

> Hematoxylin............................ 0.75 gm.
> Alcohol (96 per cent)................... 10.00 c.c.
> Water................................. 90.00 c.c.

Make fresh each time.

9. Decolorize in:

> Borax................................ 2.0 gm.
> Potassium ferricyanate................. 2.5 gm.
> Water................................. 200.0 c.c.

Dilute in portions of 1:4 or 1:10 if to be left overnight.

10. Wash thoroughly in running water.
11. Dehydrate in 80, 96 and 99 per cent alcohols.
12. Wash in carboxylol and xylol.
13. Mount in balsam.

MALLORY'S PHOSPHOTUNGSTIC ACID STAIN

Procedure.—1. Zenker's fixative is put through Lugol's 2 per cent hyposulphite solution and run down as usual to water.

2. Place in 1 per cent aqueous potassium permanganate five to fifteen minutes.

3. Place in 5 per cent oxalic acid three to five minutes.

4. Stain in phosphotungstic acid hematoxylin twelve to twenty-four hours.

> Phosphotungstic acid (Merck) (10 per cent aq. sol.) 10.0 c.c.
> Haem ammonium.. .... 0.1 gm.
> Water (distilled)............................ 90.0 c.c.

Age one to three weeks before use.

5. Clear in alcohols.
6. Dehydrate in acetone.
7. Wash in xylol.

DIETERLE METHOD FOR DEMONSTRATION OF SPIROCHETES IN TISSUES

1. After fixation and cutting, place the preparation in a 1 per cent solution of uranium nitrate in 70 per cent alcohol at 55° C. for one half hour.

2. Wash for a moment in distilled water.

3. Pass the section through 96 per cent alcohol.

4. Handling sections individually, place them in an alcoholic solution of gum mastic (10 per cent) long enough to allow infiltration (about thirty seconds).

5. Transfer to distilled water.

6. Silver for from one to six hours at 55° C. in 1 per cent aqueous solution of silver nitrate. (Carry out this procedure in exposure to light.)

7. Wash for moment in water.

8. Develop in the following reducing solution five to fifteen minutes:

Hydrochinone	1.50 gm.
Sodium sulphite....	0.25 gm.
Neutral formaldehyde.............	10.00 gm.
Acetone...............................	10.00 gm.
Pyridine	10.00 gm.
Water to make	90.00 c.c.

Mix and dissolve these and add 10 c.c. of 10 per cent absolute alcoholic mastic solution to make milky.

9. Wash for a moment in distilled water.

10. Dissolve out the mastic and dehydrate by transferring the sections to 96 per cent alcohol and then to acetone.

11. Clear in xylol.

12. Mount in balsam.

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